FORM PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C.371		**FHW-102US**
		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) **10/089922**
INTERNATIONAL APPLICATION **PCT/GB00/03792**	INTERNATIONAL FILING DATE **04 October 2000 (04.10.00)**	PRIORITY DATE CLAIMED **04 October 1999 (04.10.99)**
TITLE OF INVENTION **BINDING AGENTS**		
APPLICANT(S) FOR DO/EO/US **Rodney E. PHILLIPS, *et al.***		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C.371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). **(unexecuted) (4 Sheets);**
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98; **(3 sheets) with Form PTO-1449 (1 sheet) and reference A2 (2 sheets);**
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☒ A FIRST preliminary amendment **(6 sheets) (along with version of markings to show changes (4 sheets));**
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: **PCT International Published Application (WO 01/24829 A2) (without International Search Report attached) (64 sheets); Corrected Cover Sheet of PCT International Published Application (WO 01/24829 A3) (with International Search Report attached) (5 sheets); Certificate of First Class Mailing (1 sheet); and Return Postcard.**

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NO
10/089922	PCT/GB00/03792	FHW-102US

17. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) .(a/o November 1, 2000):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....**$1040**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.455(a)(2)) paid to USPTO **$740**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....**$710**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....**$100**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	**45**-20 =	**25**	X **$18.00**	$450.00	
Independent claims	**2**-3 =	**0**	X **$84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **280.00**	$280.00	
TOTAL OF ABOVE CALCULATIONS =				$1750.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.				$875.00	
SUBTOTAL =				$875.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$875.00	
				Amount to be: refunded	$
				charged	

a. ☐ Check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **12-0080** in the amount of $ **875.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **12-0080**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Anthony A. Laurentano, Esq.
LAHIVE & COCKFIELD, LLP
28 State Street
Boston, Massachusetts 02109
United States of America
(617)227-7400
Date: 04 April 2002

SIGNATURE

Anthony A. Laurentano
NAME

38,220
REGISTRATION NUMBER

(Atty. Docket No.: FHW-102US)

IN THE UNITED STATES PATENT DESIGNATED OFFICE (DO/US)
(National Phase of International Appln.: PCT/GB01/02543
Publication No. WO 01/98753 A1)

In re the
application of: **Rodney E. PHILLIPS et al.**

International Application No.: **PCT/GB 00/03792**

International Filing Date: **4 October 2000**

U.S. Serial No.: **Not yet assigned**

Filed: **Herewith**

For: **BINDING AGENTS**

Attorney Docket No.: **FHW-102US**

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Dear Sir:

Preliminary to examination of the above-referenced patent application, please amend the above-titled International patent application as follows:

In the Claims:

Please amend claims 1-18, 20, 21, 24, 26, 27, 29-38 as follows:

1. (Amended) A method of binding a moiety to a cell comprising, contacting a cell with a moiety at a temperature of greater than 5°C, wherein the moiety binds to a receptor on said cell that recognises an MHC peptide complex.

2. (Amended) The method according to claim 1,wherein said temperature is from 30 to 38°C.

3. (Amended) The method according to claim 1, wherein the method includes a step detecting that binding of the moiety to the cell has occurred.

4. (Amended) The method according to claim 1, wherein the temperature is lowered following and a step is included to remove cells with receptors binding to moieties at a lower specificity than binding of said moiety to said cell.

5. (Amended) The method according to claim 4, wherein said step comprises performing one or more washes.

6. (Amended) The method according to claim 1, wherein the method includes sorting cells into different types.

7. (Amended) The method according to claim 1, wherein the cell binding to the moiety is amplified.

8. (Amended) The method according to claim 1, wherein the moiety comprises an active agent.

9. (Amended) The method according to claim 8, wherein the active agent is a therapeutic agent or a diagnostic agent.

10. (Amended) The method according to claim 8, wherein the active agent is a toxin or an immunosupressant.

11. (Amended) The method according to claim 8, wherein the active agent is a label.

12. (Amended) The method according to claim 8, wherein the active agent is an agent that is active when internalised by said cell.

13. (Amended) The method according to claim 12, wherein the active agent is only active when internalised or has increased activity when internalised.

14. (Amended) The method according to claim 1, wherein said cell is a T cell.

15. (Amended) The method according to claim 14, wherein said T cell is a cytotoxic T cell.

16. (Amended) The method according to claim 1, wherein the method is performed *in vitro*.

17. (Amended) The use of a method according to claim 1 in achieving internalisation of an active agent.

18. (Amended) The use of a method according to claim 1 in screening for a therapeutically active cell.

20. (Amended) The use of a method according to claim 1 in screen for a harmful cell.

21. (Amended) The use of a method according to claim 1 in killing a harmful cell or in rendering a harmful cell less harmful.

24. (Amended) A moiety according to claim 22 which comprises a toxin molecule.

26. (Amended) A moiety according to claim 24 wherein the toxin molecule is modified so that its ability to bind with its native receptor is negligible.

27. (Amended) A moiety according to claim 25 wherein the diphtheria toxin molecule is modified by mutation of one or more amino acids at sites selected from Lys 516 or Phe 530.

29. (Amended) A moiety according to claim 27 wherein Lys 516 and Phe 530 are modified to Ala.

30. (Amended) A use of a moiety according to claim 22 in achieving internalisation of an active agent.

31. (Amended) A kit comprising a moiety as described in claim 1, wherein the kit comprises instructions to use said moiety at a temperature range of greater than 5° C or between 30° C and 38° C.

32. (Amended) An apparatus for performing a method as described in claim 1.

33. (Amended) A kit comprising an apparatus according to claim 32 and a moiety as described in claim 1.

34. (Amended) A moiety according to claim 22 for use in medicine.

35. (Amended) A method for treating a disorder involving T cells using a moiety according to claim 22.

36. (Amended) A method for treating any one of the disorders or conditions selected from the group consisting of a viral disorder, protozoan disorder, bacterial disorder, immune disorder, and a cancer using the moiety of claim 22.

37. (Amended) The method of claim 36, wherein the immune disorder is an autoimmune disorder or an undesired rejection of transplanted material.

38. (Amended) A pharmaceutical composition comprising a moiety according to claim 22.

Please cancel claim 39.

Please add claims 40-44 as follows:

40. (New) A kit comprising an apparatus according to claim 32 and a moiety as described in claim 22.

41. (New) A cell according to claim 19 for use in medicine.

42. (New) A method for treating a disorder involving T cells using a cell according to claim 19.

43. (New) A method for treating any one of the disorders or conditions selected from the group consisting of a viral disorder, protozoan disorder, bacterial disorder, immune disorder, and cancer using a cell according to claim 19.

44. (New) A pharmaceutical composition comprising a cell according to claim 19.

REMARKS

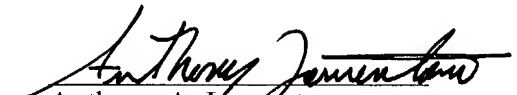
Preliminary to examination of this application, Applicant amends claims 1-18, 20, 21, 24, 26, 27, 29-38, cancels claim 39, and adds new claims 40-44 to remove multiple dependencies, to provide proper antecedent basis, and to address other matters of form. The foregoing amendments are not related to issues of patentability, and introduce no new matter.

Entry of the foregoing Preliminary Amendment is respectfully in order and requested.

If there are any questions regarding the amendments to the application, we invite the Examiner to call Applicants' representative at the telephone number below.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



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Date: **April 4, 2002**

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Please amend claims 1-18, 20, 21, 24, 26, 27, 29-39 as follows:

1. (Amended) A method ~~comprising~~ of binding a moiety to a cell comprising, contacting a cell with a moiety at a temperature of greater than 5°C, wherein the moiety binds to a receptor on said cell that recognises an MHC peptide complex.

2. (Amended) The ~~A~~ method according to claim 1,wherein said temperature is from 30 to 38°C.

3. (Amended) The ~~A~~ method according to claim 1 ~~or claim 2~~, ~~that~~ wherein the method includes a step detecting that binding of the moiety to the cell has occurred.

4. (Amended) The ~~A~~ method according to ~~any preceding~~ claim 1, wherein the temperature is lowered following and a step is included to remove cells with receptors binding to moieties at a lower specificity than binding of said moiety to said cell.

5. (Amended) The ~~A~~ method according to claim 4, wherein said step comprises performing one or more washes.

6. (Amended) The ~~A~~ method according to claim 1, wherein the method ~~any preceding claim that~~ includes sorting cells into different types.

7. (Amended) The ~~A~~ method according to ~~any preceding~~ claim 1, wherein ~~the~~ cell binding to the moiety is amplified.

8. (Amended) The ~~A~~ method according to ~~any preceding~~ claim 1, wherein the moiety comprises an active agent.

9. (Amended) The ~~A~~ method according to claim 8, wherein the active agent is a therapeutic agent or a diagnostic agent.

10. (Amended) The ~~A~~ method according to claim 8, wherein the active agent is a toxin or an immunosupressant.

11. (Amended) The ~~A~~ method according to claim 8, wherein the active agent is a label.

12. (Amended) The ~~A~~ method according to claim 8, wherein the active agent is an agent that is active when internalised by said cell.

13. (Amended) The ~~A~~ method according to claim 12, wherein the active agent is only active when internalised or has increased activity when internalised.

14. (Amended) The ~~A~~ method according to ~~any preceding~~ claim 1, wherein said cell is a T cell.

15. (Amended) The ~~A~~ method according to claim 14, wherein said T cell is a cytotoxic T cell.

16. (Amended) ~~A~~ The method according to ~~any preceding claim that~~ claim 1, wherein the method is performed *in vitro*.

17. (Amended) The use of a method according to ~~any of claims 1 to 16~~ claim 1 in achieving internalisation of an active agent.

18. (Amended) The use of a method according to ~~any of claims 1 to 16~~ claim 1 in screening for a therapeutically active cell.

20. (Amended) The use of a method according to ~~any of claims 1 to 16~~ claim 1 in screen for a harmful cell.

21. (Amended) The use of a method according to ~~any of claims 1 to 16~~ claim 1 in killing a harmful cell or in rendering a harmful cell less harmful.

24. (Amended) A moiety according to claim 22 ~~or 23~~ which comprises a toxin molecule.

26. (Amended) A moiety according to claim 24 ~~or 25~~ wherein the toxin molecule is modified so that its ability to bind with its native receptor is negligible.

27. (Amended) A moiety according to claim 25 ~~or 26~~ wherein the diphtheria toxin molecule is modified by mutation of one or more amino acids at sites selected from Lys 516 or Phe 530.

29. (Amended) A moiety according to claim 27 ~~or 28~~ wherein Lys 516 and Phe 530 are modified to Ala.

30. (Amended) A use of a moiety according to ~~any one of claims 22 to 29~~ claim 22 in achieving internalisation of an active agent.

31. (Amended) A kit comprising a moiety as described in ~~any one of claims 1 or 22 to 29~~ claim 1, wherein the kit comprises instructions to use said moiety at a temperature ~~within the range given in claim 1 or claim 2~~ range of greater than 5° C or between 30° C and 38° C.

32. (Amended) An apparatus for performing a method as described in ~~any of claims 1 to 16~~ claim 1.

33. (Amended) A kit comprising an apparatus according to claim ~~26~~32 and a moiety as described in ~~any one of claim1 or claims 22 to 29~~claim 1.

34. (Amended) A moiety according to ~~any one of claims 22 to 29, or a cell according to claim 19,~~ claim 22 for use in medicine.

35. (Amended) ~~The use of~~ A method for treating a disorder involving T cells using a moiety according to claim 22~~any one of claims 22 to 29, or a cell according to claim 19, in the preparation of a medicament for treating a disorder involving T cells~~.

36. (Amended) A method for treating any one of the disorders or conditions selected from the group consisting of ~~The use of a moiety according to any one of claims 22 to 29, or a cell according to claim 19, in the preparation of a medicament for treating~~ a viral disorder, protozoan disorder, bacterial disorder, ~~or~~ immune disorder, ~~or for treating~~ and a cancer using the moeity of claim 22.

37. (Amended) The method of ~~use according to~~ claim 36, wherein the immune disorder is an autoimmune disorder or an undesired rejection of transplanted material.

38. (Amended) A pharmaceutical composition comprising a moiety according to claim 22~~any one of claim 22 to 29, or a cell according to claim 19~~.

Please cancel claim 39.

5/prts

Binding Agents

The present invention relates to binding agents and to methods and uses in respect of such agents. More specifically, it relates to binding agents useful in targeting cells with receptors that recognise MHC peptide complexes and to methods and uses in respect of such agents.

Within the context of this specification the word "comprises" is taken to mean "includes among other things". It is not intended to be construed as "consists of only".

All documents cited herein are hereby expressly incorporated by reference.

Where preferred or optional features are described in connection with particular aspects of the present invention, they shall be deemed to apply *mutatis mutandis* to other aspects of the invention unless the context indicates otherwise.

The following documents describe moieties of multimeric complexes.

Allan, D.S., M. Colonna, L.L. Lanier, T.D. Churakova, J.S. Abrams, S.A. Ellis, A.J. McMichael, and V.M. Braud. 1999. Tetrameric complexes of human histocompatability leukocyte antigen (HLA)-G bind to peripheral blood myelomonocytic cells. *J Exp Med* 189, no. 7:1149-56;

Altman, J.D., P. Moss, P. Goulder, D.H. Barouch, W.M. McHeyzer, J.I. Bell, A.J. McMichael, and M.M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, no. 528494-6;

Anichini, A., A. Molla, R. Mortarini, G. Tragni, I. Bersani, M. Di Nicola, A.M. Gianni, S. Pilotti, R. Dunbar, V. Cerundolo, and G. Parmiani. 1999. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined,

melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. *J Exp Med* 190:651-68;

Bachmann, M.F., A. Gallimore, S. Linkert, V. Cerundolo, A. Lanzavecchia, M. Kopt, and A. Viola. 1999. Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells. *J Exp Med* 189, no. 10:1521-30;

Callan, M.F., L. Tan, N. Annels, G.S. Ogg, J.D. Wilson, C.A. O'Callaghan, N. Steven, A.J. McMichael and A.B. Rickinson. 1998. Direct visualization of antigen-specific CD8+ T Cells during the primary immune response to Epstein-Barr virus In vivo. *J Exp Med* 187, no. 9:1395-402;

Dhodapkar, M.V., R.M. Steinman, M. Sapp, H. Desai, C. Fossella J. Krasovsky, S.M. Donahoe, P.R. Dunbar, V. Cerundolo, D.F. Nixon, and N. Bhardwaj. 1999. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J Clin Invest* 104, no. 2:173-180;

Dunbar, P.R., G.S. Ogg, J. Chen, N. Rust, P. Van der Bruggen, and V. Cerundolo. 1998. Direct isolation, phenotyping, and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood. *Curr Biol* 8:413-416;

Dunbar, P.R., P. Romero, D. Valmori, M. Pittet, G.S. Ogg, D. Rimoldi, J.L. Chen, D. Lienard, J.C. Cerottini, and V. Cerundolo. 1998. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes [In Process Citation]. *J Exp Med* 188, no. 9:1641-50;

Dunbar, P.R. J.L. Chen, D. Chao, N. Rust, H. Teisserenc, G.S. Ogg, P.

Romero, P. Weynants, and V. Cerundolo. 1999. Cutting edge: rapid cloning of tumor-specific CTL suitable for adoptive immunotherapy of melanoma [In Process Citation]. *J Immunol* 162, no. 12:6959-62;

Dyer, W.B., G.S. Ogg, M.A. Demoitie, X. Jin, A.F. Geczy, S.L. Rowland-Jones, A.J. McMichael, D.F. Nixon, and J.S. Sullivan. 1999. Strong human immunodeficiency virus (HIV)-specific cytotoxic T-lymphocyte activity in Sydney Blood Bank Cohort patients infected with nef-defective HIV type 1. *J Virol* 73, no. 1:436-43;

Gallimore, A., A. Glithero, A. Godkin, A.C. Tissot, A. Pluckthun, T. Elliott, H. Hengartner, and R. Zinkernagel. 1998. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *J Exp Med* 187, no. 9:1383-93;

Gray, C.M., J. Lawrence, J.M. Schapiro, J.D. Altman, M.A. Winters, M. Crompton, M. Loi, S.K. Kundu, M.M. Davis, and T.C. Merigan. 1999. Frequency of class I HLA-restricted anti-HIV CD8+ T cells in individuals receiving highly active antiretroviral therapy (HAART). *J Immunol* 162, no. 3:1780-8;

Lee, P.P., C. Yee, P.A. Savage, L. Fong, D. Brockstedt, J.S. Weber, D. Johnson, S. Swetter, J. Thompson, P.D. Greenberg, M. Roederer, and M.M. Davis. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat Med* 5, no. 6:677-85;

McMichael, A.J., and C.A. O'Callaghan. 1998. A new look at T cells. *J Exp Med* 187, no. 9:1367-71;

Mongkolsapaya, J., A. Jaye, M.F. Callan, A.F. Magnusen, A.J. McMichael, and H.C. Whittle. 1999. Antigen-specific expansion of cytotoxic T lymphocytes in

acute measles virus infection. *J Virol* 73, no. 1:67-71;

Ogg, G.S., X. Jin, S. Bonhoeffer, P.R. Dunbar, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D.D. Ho, D.F. Nixon, and A.J. McMichael. 1998. Quantitation of HIV-I specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2103-2106;

Ogg, G.S., P.R. Dunbar, P. Romero, J.-L Chen, and V. Cerundolo. 1998. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J Exp Med* 188, no. 6:1203-1208;

Ogg, G. S., and A.J. McMichael. 1998. HLA-peptide tetrameric complexes. *Curr Opin Immunol* 10, no. 4:393-6;

Ogg, G.S., A.S. King, P.R. Dunbar, and A.J. McMichael. 1999. Isolation of human immunodeficiency virus type 1-specific cytotoxic T lymphocytes using HLA-coated beads. *AIDS* in press;

Ogg, G.S., and A.J. McMichael. 1999. Quantitation of antigen-specific CD8+ T-cell responses. *Immunol Lett* 66, no. 1-3:77-80;

Ogg, G.S., X. Jin, S. Bonhoeffer, P. Moss, M.A. Nowak, S. Monard, J.P. Segal, Y. Cao, S.L. Rowland-Jones, A. Hurley, M. Markowitz, D.D. Ho, A.J. McMichael, and D.F. Nixon. 1999. Decay kinetics of human immunodeficiency virus-specific effector cytotoxic T lymphocytes after combination antiretroviral therapy. *J Virol* 73, no. 1:797-800;

Pittet, M.J., D. Valmori, P.R. Dunbar, D.E. Speiser, D. Lienard, F. Lejeune, K. Fleischhauer, V. Cerundolo, J.-C. Cerottini, and P. Romero. 1999. High frequencies of naive melan-A/MART-1-specific CD8+ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J Exp Med*

190:705-16;

Speiser, D.E., M.J. Pittet, D. Valmori, R. Dunbar, D. Rimoldi, D. Lienard, H.R. MacDonald, J.-C. Cerottini, V. Cerundolo, and P. Romero. 1999. In vivo expression of natural killer cell inhibitory receptors by human melanoma-specific cytolytic T lymphocytes. *J Exp Med* 190, no. 6:775-782;

Spiegel, H.M., E. DeFalcon, G.S. Ogg, M. Larsson, T.J. Beadle, P. Tao, A.J. McMichael, N. Bhardwaj, C. O'Callaghan, W.I. Cox, K. Krasinski, H. Pollack, W. Borkowsky, and D.F. Nixon. 1999. Changes in frequency of HIV-1-specific cytotoxic T cell precursors and circulating effectors after combination antiretroviral therapy in children [In Process Citation]. *J Infect Dis* 180, no. 2:359-68;

Tan, L.C., N. Gudgeon, N.E. Annels, P. Hansasuta, C.A. O';Callaghan, S. Rowland-Jones, A.J. McMichael, A.B. Rickinson, and M.F. Callan. 1999. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol* 162, no. 3:1827-35;

Valmori, D., M.J. Pittet, D. Rimoldi, D. Lienard, R. Dunbar, V. Cerundolo, F. Lejeune, J.C. Cerottini, and P. Romero. 1999. An antigen-targeted approach to adoptive transfer therapy of cancer. *Cancer Res* 59, no. 9:2167-73;

Valmori, D., U. Gileadi, C. Servis, P.R. Dunbar, J.C. Cerottini, P. Romero, V. Cerundolo, and F. Levy. 1999. Modulation of proteasomal activity required for the generation of a cytotoxic T lymphocyte-defined peptide derived from the tumor antigen MAGE-3. *J Exp Med* 189, no. 6:895-906;

Yee, C., P.A. Savage, P.P. Lee, M.M. Davis, and P.D. Greenberg. 1999. Isolation of high avidity melanoma-reactive CTL from heterogenous populations using peptide-MHC tetramers. *J Immunol* 162, no. 4:2227-34.

According to one aspect of the present invention there is provided a method comprising binding a moiety to a cell at a temperature of greater than 5°C, wherein said moiety binds to a receptor present on said cell that recognises an MHC peptide complex.

Previously it has been thought that it was necessary to use low temperatures in order to achieve effective binding of such moieties to receptors. Temperatures used were about 4°C or below. It was completely unexpected to find that efficient binding can be achieved at much higher temperatures. Furthermore, the method of the present invention allows specific binding to occur to target cells at the relatively high temperatures used. This was also completely unexpected and provides a highly selective means of targeting cells expressing particular receptors. Targeting such cells has various diagnostic, therapeutic and research applications, as will be described later on.

A preferred temperature for use in the method of the present invention is at least 20°C or at least 30°C. More preferably it is from 33 to 38°C (e.g. 36 to 38°C). Most preferably it is about 37°C.

The method of the present invention may include a step of detecting the moiety once binding has occurred. Detection may be performed at or close to a temperature discussed above - i.e. without substantial cooling. Alternatively, detection may be performed at a lower temperature (e.g. after cooling on ice). In this case, it is preferred to include a step of removing or reducing non-specific binding that may occur at a lower temperature prior to performing detection. Washing can be used to remove or reduce non-specific binding.

In some circumstances it may be desired to compare specific binding occurring at a temperature of the present invention with less specific binding that may occur at lower temperatures (e.g. at 4°C or below). A method including such a comparison is within the scope of the present invention. Such a method can be used to categorise the specificity of binding of receptors to ligands (especially T cell receptors). Ligands with

high specificity may bind at both temperatures, whereas ligands of a lower specificity may bind only at the lower temperature or may not bind at all.

The method of the present invention may include a step of sorting different types of cells from one another. Techniques for cell sorting are well known and include fluorescence-based techniques, such as FACS or sorting techniques based upon magnetism. Cells that have been sorted via a method of the present invention are within the scope of the present invention. They may be used as desired or stored for later usage. They may be amplified in culture to provided homologous cell lines. Such cell lines are also within the scope of the present invention.

A moiety for use in the present invention preferably comprises an active agent. The term "active agent" refers to an agent having a desired function and also to an agent having that function only after processing by a target cell (e.g. a pro-toxin or a pro-drug). The active agent may, for example, be a therapeutically active agent or an agent useful in diagnosis or research (e.g. a label, such as a radiolabel, a fluorescence label, or a stain). It may be a biologically active molecule (e.g. a nucleic acid, cytokine, enzyme or hormone). The active agent can be associated with a part of the moiety involved in binding in a covalent or non-covalent manner. It may be present in or on a carrier (e.g. in or on a liposome).

The present inventors have surprisingly found that an active agent can be internalised by a target cell after binding of the moiety to the cell. (Prior to the present invention there was no indication that internalisation would occur). Desirably internalisation is achieved by performing binding at a temperature used for the method of the present invention. The present invention therefore includes within its scope the use of a moiety as described above in achieving internalisation of an active agent. Internalisation is particularly useful since it can remove the agent from the vicinity of non-target cells. This can provide high specificity and/or high intensity of labeling (and therefore reduce the amount of label needed). It is therefore of major importance in research and diagnostic applications. It is also of major importance in therapeutic applications, because (in addition to reducing the amount of active agent needed) it can minimise side effects associated with such applications.

The present invention therefore includes a moiety comprising a first part that binds to a receptor that recognises an MHC peptide complex and a second part comprising an agent that is capable of being internalised by a cell expressing said receptor and of being active when internalised by said cell. Moieties of the present invention may comprise any component that is desired to internalise in a target cell. Such components include therapeutic agents (e.g. toxins, immunosuppressive agents, etc), diagnostic agents, labels (e.g. fluorescent labels, radiolabels, stains etc), nucleic acid molecules, cytokines, enzymes, hormones, etc. In a preferred aspect of the invention the component requires internalisation in order to perform its function or performs its function better when internalised (e.g. it is a toxin that requires processing, such as saporin). However this is not essential.

A moiety of the present invention is preferably cell-free (prior to binding). It may be in soluble form or it may be immobilised. Immobilisation techniques are well known to those skilled in the art. They can be used to couple moieties of the present invention to an inert carrier (which is generally in solid or semi-solid form). For example, moieties of the present invention may be coupled to beads (which may be magnetic), to plates, to membranes, etc.

Preferably, one embodiment of a moiety according to the invention comprises a diphtheria toxin molecule. More preferably, it is modified so that its ability to bind with its native receptor is negligible. Preferably this ability is removed. More preferably, the diphtheria toxin molecule is modified by mutation, even more preferably at one or more amino acid sites selected from Lys 516 or Phe 530; more preferably one or more of these sites are mutated to Ala, Glu, Thr Ser or Val. Most preferably they are both mutated to Ala.

It will be appreciated that other toxin molecules could be included in addition to, or instead of, the diphtheria toxin molecule. Preferably they are modified to remove their ability to bind with their native receptors.

The present invention includes apparatuses. A moiety of the present invention may already be present in an apparatus (e.g. an apparatus for use in treatment or diagnosis) or may be introduced into an apparatus at appropriate stage. The apparatus is preferably thermostatically controlled to provide incubation at temperature at which a method of the present invention can be performed. It may include a sealed chamber in which cells can

be admixed with a moiety of the present invention. The apparatus may include means for detecting binding of said cells with a moiety of the present invention. An apparatus of the present invention may be in the form of a machine (e.g. a dialysis machine) comprising an inlet, an outlet and a chamber in which a method of the present invention is carried out. The machine may be set up to receive biological material from a patient (e.g. blood), treat it and then return it to the patient.

Having described the present invention in general terms various aspects of the present invention will now be described in further detail:

The invention has therapeutic uses.

As indicated above, the present invention is useful in therapy. Thus moieties of the present invention may be used in the manufacture of medicaments for various treatments. A medicament will usually be supplied as part of a pharmaceutical composition. The pharmaceutical composition may include a pharmaceutically acceptable carrier and can be provided in a sterile form. It may be provided in unit dosage form.

Pharmaceutical compositions within the scope of the present invention may include one or more of the following: preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts, buffers, coating agents or antioxidants. They may also contain other therapeutically active agents in addition to moetieis of the present invention.

One therapeutic use of a moiety of the present invention is that of destroying harmful cells (especially harmful T cells) or rendering them less harmful. A moiety of the present invention can therefore comprise a part capable of destroying a target cell or rendering it less harmful. Most immunotoxins are not selective, and will kill large numbers of potentially helpful T cells as well as harmful T cells they are intended to eliminate. In contrast, the present invention allows highly selective targeting of toxins to T cells (to the

level of single specificities). This can be achieved without substantial deleterious effect on other T cells. Hence the invention can allow effective treatment with reduced side effects in conditions in which T cells of given specificities are implicated. One important use is the purging of transplantation material (e.g. of bone marrow and cord blood) or potentially harmful T cells. Another important use is in the treatment of allergic conditions and of autoimmune diseases, since allergen-reactive T cells can be targeted and killed with a toxin. Alternatively, their harmful effects can be reduced by using an immunosuppressive agent.

In some cases a moiety of the present invention may exert its effect simply by binding to a receptor of a target cell binding and blocking the binding of substances needed to activate a harmful response in said cell. Such a moiety does not require an active agent, but only the ability to bind the receptor. It is preferably used to induce anergy of specific T cells, thereby inhibiting or reducing an immune response in which said T cells are implicated. The present invention includes moieties capable of doing this. For example a moiety of the present invention may comprise an immunosuppressive agent. Alternatively a moiety of the present invention can simply be used to block a T cell receptor in order to prevent stimulation of the T cell. Thus it can be used to induce anergy of specific T cells, thereby inhibiting or reducing an immune response mediated by that T cell.

The ability to inhibit immune system functions is known to be therapeutically useful in treating a variety of diseases such as atherosclerosis, allergies, autoimmune diseases, certain malignancies, arthritis, inflammatory bowel diseases, transplant rejection and reperfusion injury. Specific diseases of interest include systemic lupus erythematosus; rheumatoid arthritis; polyarteritis nodosa; polymyositis and dermatomyosis progressive systemic sclerosis (diffuse scleroderma); glomerulonephritis; myasthenia gravis; Sjogren's syndrome; Hashimoto's disease; Grave's disease; adrenalitis; hypoparathyroidism; pernicious anaemia; diabetes; multiple sclerosis, and related demyelinating diseases; uveitis; pemphigus and phempigoid cirrhosis; ulcerative colitis; myocarditis; regional enteritis; adult respiratory distress syndrome; local manifestations of drug reactions, such as dermatitis, etc., inflammation-associated or allergic reaction patterns of the skin; atopic dermatitis and infantile eczema; contact dermatitis; psoriasis; lichen planus; allergic

enteropathies; allergic rhinitis; bronchial asthma; transplant rejection, e.g. heart, kidney, lung, liver, pancreatic islet cell, etc., hypersensitivity or destructive responses to infectious agents; poststreptococcal diseases, e.g. cardiac manifestations of rheumatic fever, and the like.

Another therapeutic use of the present invention is in selecting particular cells for later use in therapy. A moiety of the present invention can be used to select particular cells from polyclonal populations of cells. Preferably it is used to select particular T cells. Selection can be performed in vitro. For example moieties of the present invention can be used to select cytotoxic T lymphocytes (CTLs) specific for particular antigens (often known as Ag-specific CTLs). It is possible to select for T cells of a single antigen specificity, even when they are rare cells found amongst many other T cells of different antigen specificity. This can be considered to be analogous to finding a needle in a haystack. This is useful in providing CTLs specific for antigens associated with various disorders - e.g. tumour, viral, bacterial or protozoan antigens. Such CTLs can be amplified if desired (e.g. by culturing the CTLs with cytokines, by using antigen stimulation, etc). The CTLs (either with or without activation) can be used in therapy - e.g. in treating cancer or in treating viral, bacterial or protozoan infection, etc.

The present invention may be used *in vivo* for various applications. However in many cases it is preferred that it is used *ex vivo*. Material can be taken from a body, treated *ex vivo* and then used as desired. For example it may be stored, it may be returned to the body or may even be put into a different body (e.g. where material from a donor is provided to a recipient). Desirably treatment *ex vivo* is performed at a temperature discussed above in relation to a method of the present invention. Instructions to use such a temperature may be included in a kit that also comprises one or moieties of the present invention. Such a kit is within the scope of the present invention. Treatment may be performed using an apparatus of the present invention. The apparatus may itself be provided in a kit together with a moiety of the present invention and optionally also together with instructions for use.

As indicated above the present invention is particularly useful if it is desired to internalise agents for use in treatment. The present invention therefore encompasses within its scope the use of moieties of the present invention in the preparation of medicaments for treating disorders via the internalisation of a therapeutic agent.

The invention has diagnostic and research uses.

The present invention is useful in diagnosis and in research. It can be used to detect cells of interest (which will usually be T cells, and will often be a specific type of T cell) in a given sample. As indicated above, greater specificity of binding (to the level of single antigen specificity) can be achieved using the present invention than is achievable using prior art methods. The present invention therefore provides greater reliability than prior art methods, since non-specific binding can be eliminated or significantly reduced. If desired cells can be quantified after binding.

The present invention is useful in characterising or diagnosing a number of conditions, especially conditions associated with T cell activation. Such conditions include autoimmune diseases, e.g. multiple sclerosis, myasthenia gravis, rheumatoid arthritis, type 1 diabetes, graft vs. host disease, Grave's disease, etc; various forms of cancer, e.g. carcinomas, melanomas, sarcomas, lymphomas and leukemia. They also include conditions involving viruses, e.g. HIV-1, hepatitis, herpes viruses, enteric viruses, respiratory viruses, rhabdovirus, rubeola, poxvirus, paramyxovirus, morbillivirus, etc. Other pathogenic conditions can also be characterised/diagnosed - e.g. conditions involving bacterial or protozoan infection. T cell associated allergic responses / autoimmune responses may also be characterised / diagnosed e.g delayed type hypersensitivity or contact hypersensitivity involving T cells.

Of particular interest are conditions having an association with a specific peptide or MHC haplotype, where the subject binding complexes may be used to track the T cell response with respect to the haplotype and antigen. A large number of associations have been made in disease states that suggest that specific MHC haplotypes, or specific protein antigens are responsible for disease states. High specificity detection can be achieved using methods of the present invention. As examples, the activity of cytotoxic T cells against HIV infected CD4+ T cells may be determined with the subject methods. The association of diabetes with the DQB1*0302 (DQ3.2) allele may be investigated by the detection and quantitation of T cells that recognize this MHC protein in combination with various peptides of interest. The presence of T cells specific for peptides of myelin basic protein in conjunction with MHC proteins of multiple sclerosis patients may be determined. The antigenic specificity may be determined for the large number of activated T cells that are found in the synovial fluid of rheumatoid arthritis patients. It will be appreciated that the subject methods are applicable to a number of diseases and immune-associated conditions.

The DNA sequence of single T cell receptors having a given antigen specificity can be determined by isolating single cells via a method of the present invention. Conveniently, flow cytometry may be used to isolate single T cells, in conjunction with single cell PCR amplification. In order to amplify unknown T cell receptor (TCR) sequences, ligation anchor PCR may be used. One amplification primer is specific for a TCR constant region. The other primer is ligated to the terminus of cDNA synthesized from TCR encoding mRNA. The variable region is amplified by PCR between the constant region sequence and the ligated primer.

In addition to the many specific diagnostic/research uses discussed above, it should be noted that the present invention is generally useful in the labelling of targeted cells (especially specific T cells). These cells may be present in biological samples (e.g. in biopsies). By using methods of the present invention labelling can be performed at higher intensity / more rapidly / with greater specificity than with prior art methods.

Having described various uses of the present invention, moieties useful in the present invention will now be described in greater detail.

As indicated above, a moiety of the present invention is capable of binding a receptor on a cell that is recognized by an MHC peptide complex. The MHC complex is preferably an MHC class I peptide complex, although it may be an MHC class II peptide complex. Desirably the MHC class I or MHC class II part of the complex is human. The peptide is preferably of non-human origin (e.g. of pathogenic origin), although in some cases it may be of human origin (e.g. if it is part of a protein associated with autoimmune diseases, cancers, etc.)

Preferably the moiety itself comprises an MHC peptide complex or a derivative thereof that retains ability to bind the receptor. The derivative can be any structure that accommodates a peptide so as to present it for recognition by a T cell receptor in the same or an analogous manner to that in which it is presented by the MHC peptide complex. Preferably the derivative comprises a grooved structure that accommodates the peptide. Such structures are well known in MHC class I and class II complexes and are shaped so as to accommodate relatively short peptides. In the case of the MHC class I complex, the peptide will usually be from about 6 to about 12 (e.g. from 6 to 8) amino acids long. In the case of the MHC Class II peptide complex the peptide will usually be from about 6 to about 20 (e.g. from 10 to 18) amino acids long. At least in the case of the MHC class I complex, the groove will generally be shaped so as to exclude peptides of lengths greater than those discussed above. Here the groove is formed between $\alpha 1$ and $\alpha 2$ domains. In the case of the MHC Class II complex, the groove is formed between $\alpha 1$ and $\alpha 2$ domains.

In moieties of the present invention it is preferred that the domains referred to above are present or that derivatives are present having substantial sequence identity therewith. (The term "derivative" is used herein to include fragments and variants. Fragments may

be of any appropriate length, but are usually at least 25% or at least 50% of the length of the relevant structure, based upon the number of amino acid residues. Variants may be naturally occurring or non-naturally occurring. They include allelic variants and also include members of the CD1 family and non-classical MHC molecules, such as HLA E).

One or more of the following (or derivatives thereof having substantial sequence identity therewith) may also be present:

a) an α3 domain comprising a disulphides bond and ß2 microglobulin (for MHC class I complexes)

b) an α2 domain and a ß2 domain (for MHC class II complexes).

As indicated above the present invention can include various derivatives having substantial sequence identity with known polypeptide structures. The term "substantial sequence identity" means at least 50% sequence identity, preferably at least 75%, at least 90% or at least 95% sequence identity. For the purposes of the present invention, sequence identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux *et al. (Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilises the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a comparison matrix (using a score of 1 for identities and 0 for non-identities), and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure,* National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Where high degrees of sequence identity are present there may be relatively few sequence differences. Thus for example there may be less than 20, less than 10, or even less than 5 differences.

There are various ways of providing moieties for use in the present invention. For example proteolytic cleavage can be used to provide soluble protein subunits from membrane-bound complexes present on cells. Alternatively these may be provided by genetic engineering techniques. The soluble units may then be associated together (after purification) in the presence of a desired peptide.

Moieties useful in the present invention include multimeric complexes of MHC protein subunits and a peptide antigen, as disclosed in WO96/26962. The uses of the complexes described in WO96/26962 apply *mutatis mutandis* to other moieties within the scope of the present invention. (It should of course be appreciated that WO96/26962 contains no disclosure of the temperatures used in the present invention for achieving high specificity binding, high intensity labeling, etc., and also that WO96/26962 does not disclose the feature of internalisation that has been found by the present inventors.)

It will be appreciated by those skilled in the art that moieties useful in the present invention include tetramers. Tetramers are well known to those skilled in the art. They are tetrameric arrays of MHC/peptide complexes. The complexes can be conjugated to avidin / streptavidin / derivatives thereof / other molecules. Biotinylation may be used in such conjugation. In the case of MHC class I complexes, an MHC class I heavy chain may be truncated of its transmembrane and cytoplasmic domain and folded by dilution around ß2 microglobulin and a peptide. In the case of MHC class II complexes, MHC class II α and ß chains may also be truncated of transmembrane and cytoplasmic domains and folded around a peptide. Here molecular links between chains may be provided (eg via a "Leucine zipper").

Tetramers are disclosed in WO96/26962 and in the papers referred to above. They can be used for the various applications of the present invention that are discussed above, including therapeutic, diagnostic and research applications. By way of example, Mutis *et al* (Nature Medicine, 5, No 7, 839-842, July 1999) disclose the labeling of T cells

involved in graft versus host (GvH) disorders. The present invention can be used to target such cells more specifically than via the methods disclosed by Mutis *et al.* It can be used to selectively kill/render less toxic harmful donor T cells involved in GvH disorders (without causing substantial damage to other T cells, some of which may be beneficial - eg in providing the beneficial graft versus leukemia effect (GvL effect). Such selectivity represents a major advance over the total T cell depletion that is often practised. It can be used to treat cells rapidly (eg in a closed system) and does not require techniques such as cell sorting.

From this description it will be appreciated that the present invention has a large number of important applications and also that many different moieties can be used in these applications. Data in support of the present invention is provided herein. The data provided is by way of example only and therefore should not be considered to be limiting.

The invention will now be described in further detail by way of examples with reference to the drawings in which

Figure 1 shows the effect of temperature on the staining of CTL cone 003 by tetramers incorporating peptide variants. The cytotoxicity and tetramer staining of CTL clone 003 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL were assessed. A, Specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of variant peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around SLYNTVATL variant peptides and the A2 HIV-1 reverse transcriptase peptide ILKEPVHGV (ILK). Staining is expressed as mean fluorescence for a homogeneous cell population and is an average of three experiments. C, Histogram plots of mean fluorescence intensity (MFI) for SL*Y* and SL*H* variant tetramer staining at 4°C, 23°C, and 37°C.

Figure 2 shows the effect of temperature on the staining of CTL clone 5D8 by tetramers incorporating peptide variants. The cytoxicity and tetramer staining of CTL clone 5D8 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL were assessed. A, Specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of various peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around variant peptides. Staining is expressed as mean fluorescence for a homogeneous cell population and is an average of two experiments.

Figure 3 shows the effect of temperature on the staining of polyclonal CTL line 868 by tetramers incorporating peptide variants. The cytotoxicity and tetramer staining of polyclonal CTL clone 868 specific for the HLA-A2.1-restricted HIV epitope SLYNTVATL were assessed. A, Specific lysis of HLA-A2.1-matched targets pulsed with 10-fold dilutions of variant peptides at an E:T ratio of 3:1. Data points represent the mean of three replicates. B, Temperature dependence of staining with tetramers folded around variant peptides. Staining with tetramer containing an HLA-A2.1-restricted epitope from HIV-1 reverse transcriptase (ILKEPVHGV) is shown for comparison. Staining is expressed as the percentage of $CD8^+$ lymphocytes visualized with tetramer for a heterogeneous cell population. C, Density plots for SI*Y* and SL*H* variant tetramer staining at 4°C, 23°C and 37°C.

Figure 4 shows timecourse of tetramer staining at 37°C. The kinetics of tetramer staining at 37°C were investigated by incubating CTL clone 003 with SLYNTVATL tetramer for various lengths of time (0.5, 5, 10 and 20 min). SLYNTVATL tetramer was not added to the "0 min" sample. The experiment was conducted in presence of 0.1% azide throughout.

Figure 5 shows that tetramers incubated at 37°C are rapidly internalized by Ag-specific CTLs. A human CTL clone specific for the HLA-A2.1-restricted IMP epitopeGILGFVFTL was incubated with FITC-labeled GILGFVFTL tetramer for 15 min at 37°C and subsequently fixed and subjected to confocal staining with and without

Texas Red costaining for the transferrin receptor. The top four panels show that when excited with the 488-nm line, only the FITC signal is detected; likewise, when excited with the 568-nm line, only the Texas Red signal is detected, confirming lack of "bleed through" between the channels. The bottom panels show excitation at 488 nm plus 568 nm, demonstrating overlap between the signals, indicating that endocytosed tetramer is reaching early endosomes that stain for transferrin receptor.

Temperature dependence and specificity of CTL interactions with peptide-MHC Class I tetrameric complexes was examined as follows.

Tetrameric peptide-MHC class I complexes ("tetramers") are proving invaluable as reagents for characterizing immune responses involved CTLs. However, because the TCR can exhibit a degree of promiscuity for binding peptide-MHC class I ligands, there is potential for cross-reactivity. Surprisingly it has now been found that tetramers rapidly stain CTLs with high intensity at 37°C. The fine specificity of tetramer staining using a well-characterized set of natural epitope variants has now been examined. Peptide variants that elicit little or no functional cellular response from CTLs can stain these cells at 4°C but not at 37°C when incorporated into tetramers. These results suggest that some studies reporting tetramer incubations at 4°C could detect cross-reactive populations of CTLs with minimal avidity for the tetramer peptide, especially in the tetramer-low population. For identifying specific CTLs among polyclonal cell populations such as PBLs, incubation with tetramers at 37°C improved the staining intensity of specific CTLs, resulting in improved separation of tetramer-high $CD8^+$ cells. Confocal microscopy revealed that tetramers incubated at 37°C can be rapidly internalized by specific CTLs into vesicles that overlap with the early endocytic compartment. This TCT-specific internalization suggested that coupling of tetramers or analogues with toxins, which are activated only after receptor internalization creates immunotoxins capable of killing CTLs of single specificities.

Tetrameric arrays of soluble peptide-MHC class I complexes ("tetramers") bind to CTLs sufficiently well to allow the labeling of CTLs according to their antigenic

specificity. Fluorogenic tetramers have already been widely used for the characterization of CTL responses and have afforded many advantages over previous techniques, particularly the ability to directly quantify and phenotype Ag-specific CTLs ex vivo. It is well documented that the TCR can exhibit a degree of promiscuity for peptide-MHC class I ligands. It has also been suggested that the enhanced avidity afforded by multimerization of peptide-MHC may allow binding to TCRs with affinities that are too low to ever generate ligand-induced physiological responses. MHC class I tetrameric complexes incorporating natural HIV-1-altered peptide ligands (APLs)[4] have now been used to investigate this possibility. Because the binding between monomeric complexes and the TCR is very weak, it had been assumed that tetrameric complexes also bind weakly to CTLs, and tetramer incubations have usually been conducted at 4°C to increase the stability of binding. Findings now reveal a temperature-dependent cross-reactivity between tetramers and low-avidity CTLs. In addition, it has now been found that tetramers stain CTLs very rapidly and with higher intensity at 37°C than at 4°C; in addition it has now been found that tetramers can be specifically internalized by CTLs.

Cell culture and cytotoxicity assays were as follows.

CTLs were derived and maintained as described previously. PBLs that had been cryopreserved were cultured for ≥2 h after thawing before staining with tetramers. Cytolytic activity was determined using standard ^{51}Cr release protocols.

Peptides were as follows.

Peptides were synthesized by standard fluorenylmethoxycarbonyl chemistry and subsequently used in chromium release assays and in synthesis of tetramers. The peptides used, referred to subsequently in the text by their first three letters only, included: SL*Y*NTVATL, an HLA-A2.1-restricted epitope from the HIV p17 gag matrix protein, and its naturally occurring variants SL*H*NTVATL, SL*S*NTVATAL, SL*F*NTVATL, SL*F*NAVATL, and SLNYT*I*AVL (24, 26, 27); ILKEPVHGV, an

HLA-A2.1-restricted epitope from the HIV pol protein (2); GLCTLVAML, an HLA-A2.1 epitope from the BMLF1 protein of EBV; and GILGFVFTL, an HLA-2.1-restricted epitope from the influenza matrix protein (IMP).

Tetrameric MHC class I/peptide complexes were as follows.

Tetrameric peptide-MHC class I complexes were made as described previously. Briefly, recombinant $ß_2$-microglobulin and the extracellular portion of the MHC class I heavy chain containing the BirA recognition sequence in frame at its C terminus were expressed in *Escherichia coli* as insoluble aggregates that formed inclusion bodies. Purified inclusion bodies were solubilized in urea, and monomeric HLA class I complexes were refolded around peptide by dilution of denaturing conditions. After buffer exchange, a specific lysine residue in the heavy chain C-terminal tag was biotinylated with BirA enzyme. Monomeric complexes were purified by gel filtration and anion exchange chromatography. Tetrameric arrays of biotinylated peptide-MHC class I complexes were formed by the addition of PE- or FITC-labeled avidin (extravidin, Sigma, S. Louis, MO). Tetramers are referred to by the first three letters of their peptide (e.g., GIL for the HLA-A2.1 GILGFVFTL tetramer).

Cell staining and FACS analysis was carried out as follows.

CTL clones and lines were washed in tissue culture medium and incubated with PE-labeled tetramers (0.1 mg/ml of monomer) at 4°C, 23°C, or 37°C for the times indicated, along with TriColor anti-human CD8 (Caltag, Burlingame, CA) when cells were double-stained. For comparison between PBL staining protocols, cells were washed in tissue culture medium before incubation with tetramers for 15 min or 60 min at 4°C or 37°C before incubation with TriColor anti-CD8 for 15 min on ice. All cells were washed twice after staining with ≥2 ml PBS/FCS 1% before fixation in 1% formaldehyde. Stained cells were analyzed by FACS (Becton Dickinson, Mountain View, CA) using CellQuest sofware.

Confocal microscopy was carried out as follows.

Cloned human CTLs specific for the HLA-A2.1-restricted IMP epitope were incubated with FITC-labeled GIL tetramer for 15 min at 37°C and subsequently adhered to glass slides coated with 50 mg/ml poly(L)-lysine (Sigma) and fixed with methanol for 5 min. Some slides were stained for 30 min with monoclonal mouse (OKT9 (anti-transferin receptor Ab), washed three times in PBS/FCS 1%, and incubated with Texas Red-conjugated goat anti-mouse sera (Jackson Laboratories, West Grove, PA) (diluted 1/100 in PBS/BSA 1% for 30 min). All slides were then washed three times as described previously and a mounted on a coverslip with PBS/glycerol 90% containing 2.5% 1,4-diazabicyclo[2,2,2]octane (Fluka, Buchs, Switzerland). Cell staining was examined with an MRC-1024 confocal microscope (Bio-Rad, Richmond, CA).

Tetramers of poorly recognised ligands stain CTL at low temperature. The results are discussed in more detail below.

The majority of HLA-A*0201 HIV-1-infected patients mount a CTL response to an immunodominant epitope (SLYNTVATL) encoded in the p17 gag matrix protein. We have documented naturally occurring mutations within this epitope that can lead to escape from, or altered recognition by, patient CTLs. CTL clones 003 and 5D8 and a CTL line from patient 868 show different patterns of recognition of naturally occurring SLYNTVATL variants (Figs. 1-3), consistent with their different origins and TCRs. We used these CTL and HLA-A2 tetramers folded around wild-type and variant peptides to examine the specificity of direct CTL visualization in vitro.

APLs, which act as weak agonist or antagonist ligands, have a lower affinity for the TCR, while they can bind to MHC with similar affinities. Tetramers of poorly recognized APLs can stain CTLs at 4°C (see SL*H* in Fig. 1, SL*S* in Fig. 2, and SL*F* in Fig. 3). Surprisingly, APL-MHC tetramers of ligands that do not stimulate CTL-mediated lysis, such as the SL*H* variant with 868 CTL, can also stain CTLs efficiently at 4°C (Fig. 3). We were unable to demonstrate antagonism of these CTLs with the

SL*H* variant. None of these A2 gag CTLs were observed to stain with A2 tetramers of the unrelated epitopes ILKEPVHGV (Figs. 1-3), GILGFVFTL, or GLCTLVAML.

Remarkably specificity for strongly recognized ligands improved with increasing temperature. This result is discussed below.

The binding of TCRs to their peptide-MHC ligands at 25°C is characterized by low affinity, slow kinetics, and a high degree of cross-reactivity. The dramatic increase in binding minetics with temperature prompted us to investigate the effect of temperature on the ability of peptide-MHC tetramers to form stable complexes with cell surface TCR. At 4°C, both CTL clones stained with tetramers containing SLYNTVATL variant peptides that they recognised only weakly in chromium relase (see variant SL*H* in Fig. 1 and SL*S* in Fig. 2). For both clones, increasing the incubation temperature to 25°C and subsequently to 37°C reduced the staining by tetramers incorporating weakly recognized peptide variants without a reduction in the strong staining by tetramers incorporating strongly recognized peptide variants (Fig. 1, *B-C*, and Fig. 2*B*0. The hierarchy of staining with peptide variant tetramers at 37°C reflected the hierarchy of recognition of each of the variant peptides in the chromium release assay (e.g., for the CTL clone 5D8 both tetramer staining and chromium release show the hierarchy SL*H*>SL*Y*>SL*S* (Fig. 2). Staining with variant tetramers at 4°C did not correlate with recognition of variant peptides in the chromium release assay for either clone.

The potential effect of this phenomenon on the staining of biological samples is seen with a polyclonal CTL line from patient 868 that also recognises SLYNTVATL peptide variants to different degrees (Fig. 3). This polyclonal line contains ~31% of SLYNTVATL-specific CTLs, as seen by staining with the SL*Y* tetramer (Fig. 3*B*). When incubated at 4°C, tetramers made from the SL*H* variant peptide, which does not stimulate CTL-mediated lysis, stain a population of similar size (Fig. 3*B*). Increasing the temperature of the tetramer incubation reduces the proportion of this line that stains with the SL*H* tetramer, so that at 37°C, there is no staining with this unrecognized

peptide variant (Fig. 3, *B-C*). The results for three different TCRs and several APLs in Figs. 1-3 show that improvements in staining specificity for strongly recognised ligands with increasing temperature are neither TCR- nor APL-dpendent.

In addition to increased specificity for well-recognised peptide variants, staining at 37°C also increased staining intensity with strongly recognised ligands (see the SL*Y* tetramer in Fig. 1*B* aand the SL*H* tetramer in Fig. 2*B*).

It is important to note that all SLYNTVATL variant tetramers used are sufficiently stable at 37°C to stain appropriate CTLs brightly (Fig. 1-2). Hence where increasing temperature reduces tetramer staining, in the case of variant peptides that are poorly recognised by the CTLs, this is not due to tetramer instability at higher temperature (e.g. the SL*F* tetramer stains 003 CTLs brightly at 37°C but does not stain 868 CTLs at this temperature). Preincubation of tetramers at 37°C for 60 min before staining CTLs also had no demonstrable effects on subsequent staining (data not shown), confirming the stability of the reagents under the experimental conditions employed.

Tetramers can be internalised by CTL at 37°C. This important result is discussed below.

Incubation of the 003 CTL clone (Fig. 4) and the 868 CTL line (data not shown) for between 0 min and 20 min at 37°C showed that staining was complete after 5 min and did not increase significantly with longer incubations. Staining occurred in minutes in the presence of 0.1% azide during staining (Fig. 4) and was indistinguishable from staining without the inclusion of azide (data not shown).

The rapid bright stable staining observed of CTLS at 37°C led us to examine whether tetramers were being internalized after interacting with the TCR. An IMP-specific CTL clone that stained in the FACS analysis with GILGFVFTL tetramer but not with other HLA-A2 tetramers was incubated with this tetramer for 15 min at 37°C and examined by confocal microscopy, with and without double staining, to identify

intracellular compartments. Tetramer was indeed internalised by this clone within 15 min at 37°C, and was visible in intracellular vesicles (Fig. 5). These vesicles overlapped in distribution with vesicles containing the transferrin receptor, although not all of these early endosomes were labeled with tetramer. Internalized tetramer did not colocalize to secretory lysosomes containing perforin or granzyme B. Incubation with tetramer for 15 min at 4°C did not induce detectable internalisation of tetramer.

In view of the results obtained it seems that peptide-MHC class I tetramers are set to revolutionize the analysis of CTL responses and have already been used in a wide variety of pathological conditions. Tetramers have commonly revealed surprisingly high frequencies of Ag-specific CTLs compared with those estimated by previous techniques. This descrepancy is probably due in large part to the technical limitations imposed by other methods. The detection of specific CTLs by limiting dilution analysis, for example, requires high profilerative potential, which probably explains why the frequencies obtained are approximately one order of magnitude lower than those revealed by tetramers. Neverthless, because it is well documented that the TCR can exhibit a degree of promiscuity for peptide-MHC class I ligands, there is significant potential for cross-reactivity in the identification of Ag-specific CTLs using tetramers.

Peptide-MHC complexes need not bind with high affinity to trigger the TCR. A recent report shows that there is little correlation between the equilibrium binding affinity (K_d) of a ligand and its potency for T cell activation, and that the potency of a ligand correlates much better with its off-rate. Small changes in the squence of an antigenic peptide (APL) can lead to slightly faster off-rates; relatively small changes in the off-rate can greatly affect the ability of a ligand to trigger T cells. To activate T cells, a single peptide-MHC needs to serially trigger multiple TCRs. Thus, strongly recognised ligands fall within a "kinetic window" representing a compromise between enough time to allow all the molecular steps required to trigger a TCR and a rapid enough dissociation from the triggered TCR to make it available for further cycles of binding and triggering. Assuming a fast enough on-rate, the ability of peptide-MHC

class I tetramers to stain CTLs for FACScan analysis will be exclusively dependent upon a sufficiently slow off-rate. Our data demonstrate that the dramatic temperature dependence of the TCR/peptide-MHC interaction can be exploited to increase the specificity of tetramer staining for strongly recognized ligands. At higher temperatures, it appears that the off-rate can be increased beyond the threshold at which the avidity of tetrameric forms of weakly recognised MHC ligands can compensate to allow the formation of stable complexes with cell surface TCRs.

Moderate changes in peptide sequence may allow sufficient interaction at low temperature for formation of stable complexes with cell surface TCR when tetrmerized but fall outside the kinetic window for activation. This appears to be the case for the [^{3}H] variant with the 868 CTL (Fig. 3). Consequently, the use of tetramers to stain CTLs within PBL samples at a low temperature may result in background staining due to the binding of tetramers to TCRs that only recognise the peptide-MHC very weakly. This may result in an overestimation of the frequency of functional Ag-specific CTLs, especially when tetramer-low cells are counted as tetramer-positive. Importantly, however, we have no evidence that CTLs will bind tetramers incorporating completely unrelated peptides, even at 4°C. All clones and polyclonal lines reported here showed no background staining with tetramers based around any other epitope, confirming that tetramers demonstrate a high specificity for CTLs recognizing their peptide-MHC class I complex.

Although the cross-reactivity of tetramers at 4°C probably relates to only fine differences in CTL specificity for highly similar epitopes, the effects of this phenomenon on tetramer-generated data may be potentially important. The possibility of cross-staining with tetramers is of most concern with pathogens of variable antigenicity such as HIV-1 or hepatitis C virus. The phenomenon of original antigenic sin or epitope imprinting, whereby the exposure to an Ag influences subsequent responses to similar Ags, was recently extended to CTL epitopes. This study showed that mice primed with lymphocyte choriomeningitis virus respond to a subsequent infection by lymphocyte choriomeningitis virus containing CTL epitope variants with

a CTL response directed against the initial epitope rather than against the new variant. Maintenance of an initial CTL response in the face of a poorly recognised variant CTL epitope could severely compromise the effectiveness of the overall CTL response. It was noted that pathogens of variable antigenicity may exploit this phenomenon. The incubation of tetramers at both 4°C and 37°C may allow distinction between CTLs that recognise variant epitopes to different degrees and may prove to be a useful tool for tracking Ctl responses as epitopes mutate.

Cross-reactive staining may be problematic in other systems. When Yee *et al* used tetramer incubations at 4°C to study CTLs specific for the melanoma Ag Melan-A, tetramer-low CTLs lysed peptide-pulsed cells but had insufficient avidity to lyse tumor cells. Substitution of a brief 37°C incubation for a longer 4°C incubation reduces the detection of this potentially nontumoricidal tetramer-low population relative to the highly lytic tetramer-high population. Such effects may be particularly important for this HLA-A2.1-restricted Melan-A_{26-35} epitope, where CTLs specific for epitopes from a number of common viruses may cross-react with the Melan-A peptide. Given the huge array of nonself peptides to which the human immune system must be exposed, such epitope mimicry may not be an uncommon phenomenon. Although staining at 4°C may not be problematic in many systems, our results suggest that cross-reactive tetramer staining of CTLs specific for other epitopes that may closely mimic the epitope of interest will be lower at 37°C. If performing low temperature staining, inclusion of a high temperature control and confirmation of Ag specificity after cell sorting are recommended. Exploitation of our findings to isolate both high- and low-avidity CTLs may prove useful to some researchers.

In addition to improvements in specificity, we have demonstrated that staining at 37°C consistently produces higher intensity staining of both clones (Figs. 1-2) and Ag-specific CTLs within polyclonal populations, and allows clearer separation of these cells from tetramer-negative cells (data not shown). Interestingly, our findings of tetramer internalisation at 37°C suggest an important new therapeutic possibility. Because tetramers show such fine specificity for high-avidity CTLs at 37°C,

multimerized peptide-MHC class I complexes may make highly specific probes for targeting CTLs in vivo. Internalization of these probes might allow toxins to be carried to the intracellular compartments of CTLs with very high specificity. In particular, toxins such as saporin which are biologically active only when internalized through recepto-mediated internalization can be conjugated onto multimerized peptide-MHC class I complexes or similar structures to allow specific immunolysis of CTLs involved in pathology such as autoimmune diseases.

The results obtained may be broadly relevant to all studies using peptide-MHC class I tetrameric complexes to quantify CTLs ex vivo. If the interaction between peptide-MHC class II and the TCR is also influenced by temperature, the similar considerations will likely apply to the use of tetramer technology in MHC class-II-restricted systems.

Further specific embodiments of binding agents useful in targeting cells with receptors that recognise MHC peptide complexes are described below.

Compositions are provided that label T cells according to the specificity of their antigen receptor. A stable multimeric complex of MHC protein subunits and peptide antigen is prepared. The specificity of the multimeric binding complex is determined both by the antigenic peptide and the MHC protein. The binding complex binds stably with the antigen receptor on the surface of T cells, allowing the detection of antigen specific T cells. The binding complex is useful for detection, quantitation, characterization and separation of specific T cells. Variants of the binding complex for different MHC proteins and peptide antigens are easily produced.

The T cell receptor (TCR) specificity determines what antigens will activate that particular T cell. Usually T helper cells express CD4 on their surface, and are activated by binding to a complex of antigenic peptide and Class II MHC molecule. Usually cytolytic T cells express CD8 on their surface, and are activated by binding to a complex of antigenic peptide and Class I MHC molecule. The specificity of the T cell antigen receptor is the combination of peptide and MHC molecule that binds to that particular TCR with sufficient affinity to activate the T cell. The binding affinity will usually be at least about 10^{-4} M, as described in Matsui *et al.*, *supra*.

The binding complex may have a wide variety of peptide-MHC combinations. Multimers of class I MHC molecules will usually be used to detect $CD8^+$ T cells, and class II multimers will usually be used to detect $CD4^+$ T cells. Quantitation of T cells may be performed to monitor the progression of a number of conditions associated with T cell activation, including autoimmune diseases, graft rejection, viral infection, bacterial and protozoan infection. T cells having a particular antigenic specificity may be separated from complex mixtures, particularly biological samples, through binding

to the binding complexes. In this way selective depletion or enrichment of particular T cells can be made.

The multimeric binding complex has the formula $(\alpha-\beta-P)_n$, where $n \geq 2$, usually $n \geq 4$, and usually $n \leq 10$. α is an α chain of a class I or class II MHC protein. β is a β chain, herein defined as the β chain of a class II MHC protein or β_2 microglobulin for a MHC class I protein. P is a peptide antigen. The multimeric complex stably binds through non-covalent interactions to a T cell receptor having the appropriate antigenic specificity. When compared to the binding of an $(\alpha-\beta-P)$ "monomer" to a T cell, the binding complex will have greatly increased stability, usually having an increase of at least about 10-fold in $t_{1/2}$, more usually an increase of about 20-fold, and may be increased as much as about 50-fold.

The MHC proteins may be from any mammalian or avian species, *e.g.* primate *sp.*, particularly humans; rodents, including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; *etc*. Of particular interest are the human HLA proteins, and the murine H-2 proteins. Included in the HLA proteins are the class II subunits HLA-DPα, HLA-DPβ, HLA-DQα, HLA-DQβ, HLA-DRα and HLA-DRβ, and the class I proteins HLA-A, HLA-B, HLA-C, and β_2-microglobulin. Included in the murine H-2 subunits are the class I H-2K, H-2D, H-2L, and the class II I-Aα, I-Aβ, I-Eα and I-Eβ, and β_2-microglobulin. Sequences of some representative MHC proteins may be found in Kabat *et al.* Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242, pp724-815.

In a preferred embodiment, the MHC protein subunits are a soluble form of the normally membrane-bound protein. The soluble form is derived from the native form by deletion of the transmembrane domain. Conveniently, the protein is truncated, removing both the cytoplasmic and transmembrane domains. The protein may be truncated by proteolytic cleavage, or by expressing a genetically engineered truncated form. As a reference, the alignment of amino acids into domains shown in Kabat *et al.*, *ibid.* will be used.

For class I proteins, the soluble form will include the α1, α2 and α3 domain. Not more than about 10, usually not more than about 5, preferably none of the amino acids of the transmembrane domain will be included. The deletion may extend as much as about 10 amino acids into the α3 domain, preferably none of the amino acids of the α3 domain will be deleted. The deletion will be such that it does not interfere with the ability of the α3 domain to fold into a disulfide bonded structure. The class I β chain, β_2-microglobulin, lacks a transmembrane domain in its native form, and need not be truncated. Generally, no Class II subunits will be used in conjunction with Class I subunits.

Soluble class II subunits will include the α1 and α2 domains for the α subunit, and the β1 and β2 domains for the β subunit. Not more than about 10, usually not more than about 5, preferably none of the amino acids of the transmembrane domain will be included. The deletion may extend as much as about 10 amino acids into the α2 or β2 domain, preferably none of the amino acids of the α2 or β2 domain will be deleted. The deletion will be such that it does not interfere with the ability of the α2 or β2 domain to fold into a disulfide bonded structure.

One may wish to introduce a small number of amino acids at the polypeptide termini, usually not more than 20, more usually not more than 15. The deletion or insertion of amino acids will usually be as a result of the needs of the construction, providing for convenient restriction sites, addition of procesing signals, ease of manipulation, improvement in levels of expression, or the like. In addition, one may wish to substitute one or more amino acids with a different amino acid for similar reasons, usually not substituting more than about five amino acids in any one domain.

The α and β subunits may be separately produced and allowed to associate *in vitro* to form a stable heteroduplex complex (see Altman *et al.* [1993], *supra.* or Garboczi *et al.* (1992) *supra.*), or both of the subunits may be expressed in a single cell. An alternative strategy is to engineer a single molecule having both the α and β subunits. A "single-chain heterodimer" is created by fusing together the two subunits

using a short peptide linker, *e.g.* a 15 to 25 amino acid peptide or linker. See Bedzyk *et al.* (1990) J. Biol. Chem. 265:18615 for similar structures with antibody heterodimers. The soluble heterodimer may also be produced by isolation of a native heterodimer and cleavage with a protease, *e.g.* papain, to produce a soluble product.

In a preferred embodiment, soluble subunits are independently expressed from a DNA construct encoding a truncated protein. For expression, the DNA sequences are inserted into an appropriate expression vector, where the native transcriptional initiation region may be employed or an exogenous transcriptional initiation region, *i.e.* a promoter other than the promoter which is associated with the gene in the normally occurring chromosome. The promoter may be introduced by recombinant methods *in vitro*, or as the result of homologous integration of the sequence into a chromosome. A wide variety of transcriptional initiation regions are known for a wide variety of expression hosts, where the expression hosts may involve prokaryotes or eukaryotes, particularly *E. coli*, *B. subtilis*, mammalian cells, such as CHO cells, COS cells, monkey kidney cells, lymphoid cells, particularly human cell lines, and the like. Generally a selectable marker operative in the expression host will be present.

Of particular interest are expression cassettes comprising a transcription initiation region, the gene encoding the subject MHC subunit, and a transcriptional termination region, optionally having a signal for attachment of a poly A sequence. Suitable restriction sites may be engineered into the termini of the MHC subunit, such that different subunits may be put into the cassette for expression . Restriction sites may be engineered by various means, *e.g.* introduction during polymerase chain reaction, site directed mutagenesis, *etc.*

The subunits are expressed in a suitable host cell, and, if necessary, solubilized. The two subunits are combined with an antigenic peptide and allowed to fold *in vitro* to form a stable heterodimer complex with intrachain disulfide bonded domains. The peptide may be included in the initial folding reaction, or may be added to the empty heterodimer in a later step. Usually the MHC binding site will be free of

peptides prior to addition of the target antigenic peptide. The exception will be those cases where it is desirable to label the T cells with a natural peptide-MHC complex, such as those that may be present on the surface of cells that are a target for autoimmune attack, *etc.*

The MHC heterodimer will bind an antigenic peptide in the groove formed by the two membrane distal domains, either α2 and α1 for class I, or α1 and β1 for class II. The bound peptide will be substantially homogenous, that is, there will be less than about 10% of the bound peptides having an amino acid sequence different from the desired sequence, usually less than about 5%, and more usually less than about 1%.

Conditions that permit folding and association of the subunits and peptide are known in the art, see for example Altman *et al.* (1993) and Garboczi *et al.* (1992). As one example of permissive conditions, roughly equimolar amounts of solubilized α and β subunits are mixed in a solution of urea. Refolding is initiated by dilution or dialysis into a buffered solution without urea. Peptides are loaded into empty class II heterodimers at about pH 5 to 5.5 for about 1 to 3 days, followed by neutralyzation, concentration and buffer exchange. However, it will be readily understood by one of skill in the art that the specific folding conditions are not critical for the practice of the invention.

The antigenic peptide will be from about 6 to 12 amino acids in length for complexes with class I MHC proteins, usually from about 8 to 10 amino acids. The peptide will be from about 6 to 20 amino acids in length for complexes with class II MHC proteins, usually from about 10 to 18 amino acids. The peptides may have a sequence derived from a wide variety of proteins. In many cases it will be desirable to use peptides which act as T cell epitopes. The epitopic sequences from a number of antigens are known in the art. Alternatively, the epitopic sequence may be empirically determined, by isolating and sequencing peptides bound to native MHC proteins, by synthesis of a series of peptides from the target sequence, then assaying for T cell reactivity to the different peptides, or by producing a series of binding complexes with

different peptides and quantitating the T cell binding. Preparation of fragments, identifying sequences, and identifying the minimal sequence is amply described in U.S. Patent No. 5,019,384, iss. 5-28-91, and references cited therein.

The peptides may be prepared in a variety of ways. Conveniently, they can be synthesized by conventional techniques employing automatic synthesizers, or may be synthesized manually. Alternatively, DNA sequences can be prepared which encode the particular peptide and may be cloned and expressed to provide the desired peptide. In this instance a methionine may be the first amino acid. In addition, peptides may be produced by recombinant methods as a fusion to proteins that are one of a specific binding pair, allowing purification of the fusion protein by means of affinity reagents, followed by proteolytic cleavage, usually at an engineered site to yield the desired peptide (see for example Driscoll et al. (1993) J. Mol. Bio. 232:342-350). The peptides may also be isolated from natural sources and purified by known techniques, including, for example, chromatography on ion exchange materials, separation by size, immunoaffinity chromatography and electrophoresis.

The monomeric complex (α–β–P) (herein monomer) is multimerized. The resulting multimer will be stable over long periods of time. Usually not more than about 10% of the multimer will be dissociated after storage at 4°C for about one day, more usually after about one week. Preferably, the multimer will be formed by binding the monomers to a multivalent entity through specific attachment sites on the α or β subunit, as described below in detail. The multimer may also be formed by chemical cross-linking of the monomers. A number of reagents capable of cross-linking proteins are known in the art, illustrative entities include: azidobenzoyl hydrazide, N-[4-(p-azidosalicylamino)butyl]-3'-[2'-pyridyldithio]propionamide), bis-sulfosuccinimidyl suberate, dimethyladipimidate, disuccinimidyltartrate, N-γ-maleimidobutyryloxysuccinimide ester, N-hydroxy sulfosuccinimidyl-4-azidobenzoate, N-succinimidyl [4-azidophenyl]-1,3'-dithiopropionate, N-succinimidyl

[4-iodoacetyl]aminobenzoate, glutaraldehyde, formaldehyde and succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate.

The attachment site for binding to a multivalent entity may be naturally occurring, or may be introduced through genetic engineering. The site will be a specific binding pair member or one that is modified to provide a specific binding pair member, where the complementary pair has a multiplicity of specific binding sites. Binding to the complementary binding member can be a chemical reaction, epitope-receptor binding or hapten-receptor binding where a hapten is linked to the subunit chain. In a preferred embodiment, one of the subunits is fused to an amino acid sequence providing a recognition site for a modifying enzyme. The recognition sequence will usually be fused proximal to the carboxy terminus of one of the subunit to avoid potential hindrance at the antigenic peptide binding site. Conveniently, an expression cassette will include the sequence encoding the recognition site.

Modifying enzymes of interest include BirA, various glycosylases, farnesyl protein transferase, protein kinases and the like. The subunit may be reacted with the modifying enzyme at any convenient time, usually after formation of the monomer. The group introduced by the modifying enzyme, *e.g.* biotin, sugar, phosphate, farnesyl, *etc.* provides a complementary binding pair member, or a unique site for further modification, such as chemical cross-linking, biotinylation, *etc.* that will provide a complementary binding pair member. An alternative strategy is to introduce an unpaired cysteine residue to the subunit, thereby introducing a unique and chemically reactive site for binding. The attachment site may also be a naturally occurring or introduced epitope, where the multivalent binding partner will be an antibody, *e.g.* IgG, IgM, *etc.* Any modification will be at a site, *e.g.* C-terminal proximal, that will not interfere with binding.

Exemplary of multimer formation is the introduction of the recognition sequence for the enzyme BirA, which catalyzes biotinylation of the protein substrate. The monomer with a biotinylated subunit is then bound to a multivalent binding partner,

e.g. streptavidin or avidin, to which biotin binds with extremely high affinity. Streptavidin has a valency of 4, providing a multimer of $(\alpha-\beta-P)_4$.

The multivalent binding partner may be free in solution, or may be attached to an insoluble support. Examples of suitable insoluble supports include beads, *e.g.* magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (*e.g.* polystyrene), polysaccharides, nylon or nitrocellulose. Attachment to an insoluble support is useful when the binding complex is to be used for separation of T cells.

Frequently, the multimeric complex will be labeled, so as to be directly detectable, or will be used in conjunction with secondary labeled immunoreagents which will specifically bind the complex. In general the label will have a light detectable characteristic. Preferred labels are fluorophors, such as fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin and allophycocyanin. Other labels of interest may include dyes, enzymes, chemiluminescers, particles, radioisotopes, or other directly or indirectly detectable agent. Conveniently, the multivalent binding partner will have the labeling group. Alternatively, a second stage label may be used, *e.g.* labeled antibody directed to one of the peptide constituents, and the like.

The binding complex will be used to detect and/or separate antigen specific T cells. The T cells may be from any source, usually having the same species of origin as the MHC heterodimer. The T cells may be from an *in vitro* culture, or a physiologic sample. For the most part, the physiologic samples employed will be blood or lymph, but samples may also involve other sources of T cells, particularly where T cells may be invasive. Thus other sites of interest are tissues, or associated fluids, as in the brain, lymph node, neoplasms, spleen, liver, kidney, pancreas, tonsil, thymus, joints, synovia, and the like. The sample may be used as obtained or may be subject to modification, as in the case of dilution, concentration, or the like. Prior treatments may involve removal of cells by various techniques, including

centrifugation, using Ficoll-Hypaque, panning, affinity separation, using antibodies specific for one or more markers present as surface membrane proteins on the surface of cells, or any other technique that provides enrichment of the set or subset of cells of interest.

The binding complex is added to a suspension comprising T cells of interest, and incubated at about 4°C for a period of time sufficient to bind the available cell surface receptor. The incubation will usually be at least about 5 minutes and usually less than about 30 minutes. It is desirable to have a sufficient concentration of labeling reagent in the reaction mixture, so that labeling reaction is not limited by lack of labeling reagent. The appropriate concentration is determined by titration. The medium in which the cells are labeled will be any suitable medium as known in the art. If live cells are desired a medium will be chosen that maintains the viability of the cells. A preferred medium is phosphate buffered saline containing from 0.1 to 0.5% BSA. Various media are commercially available and may be used according to the nature of the cells, including Dulbecco's Modified Eagle Medium (dMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (dPBS), RPMI, Iscove's medium, PBS with 5 mM EDTA, *etc.*, frequently supplemented with fetal calf serum, BSA, HSA, *etc.*

Where a second stage labeling reagent is used, the cell suspension may be washed and resuspended in medium as described above prior to incubation with the second stage reagent. Alternatively, the second stage reagent may be added directly into the reaction mix.

A number of methods for detection and quantitation of labeled cells are known in the art. Flow cytometry is a convenient means of enumerating cells that are a small percent of the total population. Fluorescent microscopy may also be used. Various immunoassays, *e.g.* ELISA, RIA, *etc.* may used to quantitate the number of cells present after binding to an insoluble support.

Flow cyometry may also be used for the separation of a labeled subset of T cells from a complex mixture of cells. The cells may be collected in any appropriate medium which maintains the viability of the cells, usually having a cushion of serum at the bottom of the collection tube. Various media are commercially available as described above. The cells may then be used as appropriate.

Alternative means of separation utilize the binding complex bound directly or indirectly to an insoluble support, *e.g.* column, microtiter plate, magnetic beads, *etc.* The cell sample is added to the binding complex. The complex may be bound to the support by any convenient means. After incubation, the insoluble support is washed to remove non-bound components. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound cells present in the sample. The desired cells are then eluted from the binding complex. In particular the use of magnetic particles to separate cell subsets from complex mixtures is described in Miltenyi *et al.* (1990) Cytometry 11:231-238.

Detecting and/or quantitatingspecific T cells in a sample or fraction thereof may be accomplished by a variety of specific assays. In general, the assay will measure the binding between a patient sample, usually blood derived, generally in the form of plasma or serum and the subject multimeric binding complexes. The patient sample may be used directly, or diluted as appropriate, usually about 1:10 and usually not more than about 1:10,000. Assays may be performed in any physiological buffer, *e.g.* PBS, normal saline, HBSS, dPBS, *etc.*

A sandwich assay is performed by first attaching the multimeric binding complex to an insoluble surface or support. The the multimeric binding complex may be bound to the surface by any convenient means, depending upon the nature of the surface, either directly or through specific antibodies. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which the multimeric binding complex can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method of measuring T cells. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, *e.g.* magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Before adding patient samples or fractions thereof, the non-specific binding sites on the insoluble support *i.e.* those not occupied by the multimeric binding complex, are generally blocked. Preferred blocking agents include non-interfering proteins such as bovine serum albumin, casein, gelatin, and the like. Samples, fractions or aliquots thereof are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing support-bound multimeric binding complex.

Generally from about 0.001 to 1 ml of sample, diluted or otherwise, is sufficient, usually about 0.01 ml sufficing. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for T cells to bind the insoluble binding complex. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute physiologic buffer at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound T cells present in the sample.

After washing, a solution containing specific second receptor is applied. The receptor may be any compound that binds patient T cells with sufficient specificity such

that they can be distinguished from other components present. In a preferred embodiment, second receptors are antibodies specific for common T cell antigens, either monoclonal or polyclonal sera, *e.g.* anti-thy-1, anti-CD45, *etc.*

T cell specific antibodies may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement include radiolabels, such as ^{3}H or ^{125}I, fluorescers, dyes, beads, chemilumninescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art.

Alternatively, the second receptor may be unlabeled. In this case, a labeled second receptor-specific compound is employed which binds to the bound second receptor. Such a second receptor-specific compound can be labelled in any of the above manners. It is possible to select such compounds such that multiple compounds bind each molecule of bound second receptor. Examples of second receptor/second receptor-specific molecule pairs include antibody/anti-antibody and avidin (or streptavidin)/biotin. Since the resultant signal is thus amplified, this technique may be advantageous where only a small number of T cells are present. An example is the use of a labeled antibody specific to the second receptor. More specifically, where the second receptor is a rabbit anti-allotypic antibody, an antibody directed against the constant region of rabbit antibodies provides a suitable second receptor specific molecule. The anti-immunoglobulin will usually come from any source other than human, such as ovine, rodentia, particularly mouse, or bovine.

The volume, composition and concentration of T cell specific receptor solution provides for measurable binding to the T cells already bound to the insoluble substrate. Generally, the same volume as that of the sample is used: from about 0.001 to 1 ml is

sufficient, usually about 0.1 ml sufficing. When antibody ligands are used, the concentration generally will be about 0.1 to 50 μg/ml, preferably about 1 μg/ml. The solution containing the second receptor is generally buffered in the range of about pH 6.5-9.5. The solution may also contain an innocuous protein as previously described. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second receptor or second receptor-conjugate has bound, the insoluble support is generally again washed free of non-specifically bound second receptor, essentially as described for prior washes. After non-specifically bound material has been cleared, the signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed. More specifically, where a peroxidase is the selected enzyme conjugate, a preferred substrate combination is H_2O_2 and is O-phenylenediamine which yields a colored product under appropriate reaction conditions. Appropriate substrates for other enzyme conjugates such as those disclosed above are known to those skilled in the art. Suitable reaction conditions as well as means for detecting the various useful conjugates or their products are also known to those skilled in the art. For the product of the substrate O-phenylenediamine for example, light absorbance at 490-495 nm is conveniently measured with a spectrophotometer.

Generally the number of bound T cells detected will be compared to control samples from samples having a different MHC context, e.g. T cells from an animal that does not express the MHC molecule used to make the binding complex.

An alternative protocol is to provide anti-T cell reagent, *e.g.* anti-thy-1, anti-CD45, *etc.* bound to the insoluble surface. After adding the sample and washing away non-specifically bound T cells, one or a combination of the subject binding complexes are added, where the binding complexes are labeled so as not to interfere with the binding to T cells.

It is particularly convenient in a clinical setting to perform the assays in a self-contained apparatus. A number of such methods are known in the art. The apparatus will generally employ a continuous flow-path of a suitable filter or membrane, having at least three regions, a fluid transport region, a sample region, and a measuring region. The sample region is prevented from fluid transfer contact with the other portions of the flow path prior to receiving the sample. After the sample region receives the sample, it is brought into fluid transfer relationship with the other regions, and the fluid transfer region contacted with fluid to permit a reagent solution to pass through the sample region and into the measuring region. The measuring region may have bound to it the multimeric binding complex, with a conjugate of an enzyme with T cell specific antibody employed as a reagent, generally added to the sample before application. Alternatively, the binding complex may be conjugated to an enzyme, with T cell specific antibody bound to the measurement region.

Detection of T cells is of interest in connection with a variety of conditions associated with T cell activation. Such conditions include autoimmune diseases, *e.g.* multiple sclerosis, myasthenia gravis, rheumatoid arthritis, type 1 diabetes, graft vs. host disease, Grave's disease, *etc.*; various forms of cancer, *e.g.* carcinomas, melanomas, sarcomas, lymphomas and leukemias. Various infectious diseases such as those caused by viruses, *e.g.* HIV-1, hepatitis, herpesviruses, enteric viruses, respiratory viruses, rhabdovirus, rubeola, poxvirus, paramyxovirus, morbillivirus, *etc.* are of interest. Infectious agents of interest also include bacteria, such as *Pneumococcus, Staphylococcus, Bacillus, Streptococcus, Meningococcus, Gonococcus, Eschericia, Klebsiella, Proteus, Pseudomonas, Salmonella, Shigella, Hemophilus, Yersinia, Listeria, Corynebacterium, Vibrio, Clostridia, Chlamydia, Mycobacterium, Helicobacter* and *Treponema*; protozoan pathogens, and the like. T cell associated allergic responses may also be monitored, *e.g.* delayed type hypersensitivity or contact hypersensitivity involving T cells.

Of particular interest are conditions having an association with a specific peptide or MHC haplotype, where the subject binding complexes may be used to track the T cell response with respect to the haplotype and antigen. A large number of associations have been made in disease states that suggest that specific MHC haplotypes, or specific protein antigens are responsible for disease states. However, direct detection of reactive T cells in patient samples has not been possible. Detection and quantitation with the subject binding complexes allows such direct detection. As examples, the activity of cytolytic T cells against HIV infected CD4+ T cells may be determined with the subject methods. The association of diabetes with the DQB1*0302 (DQ3.2) allele may be investigated by the detection and quantitation of T cells that recognize this MHC protein in combination with various peptides of interest. The presence of T cells specific for peptides of myelin basic protein in conjunction with MHC proteins of multiple sclerosis patients may be determined. The antigenic specificity may be determined for the large number of activated T cells that are found in the synovial fluid of rheumatoid arthritis patients. It will be appreciated that the subject methods are applicable to a number of diseases and immune-associated conditions.

The isolation of antigen specific T cells finds a wide variety of applications. The isolated T cells cells may find use in the treatment of cancer as in the case of tumor-infiltrating lymphocytes. Specific T cells may be isolated from a patient, expanded in culture by cytokines, antigen stimulation, *etc.*, and replaced in the autologous host, so as to provide increased immunity against the target antigen. A patient sample may be depleted of T cells reactive with a specific antigen, to lessen an autoimmune response.

The DNA sequence of single T cell receptors having a given antigen specificity is determined by isolating single cells by the subject separation method. Conveniently, flow cytometry may be used to isolate single T cells, in conjunction with single cell PCR amplification. In order to amplify unknown TCR sequences, ligation anchor PCR may be used. One amplification primer is specific for a TCR constant region. The other primer is ligated to the terminus of cDNA synthesized from TCR encoding

mRNA. The variable region is amplified by PCR between the constant region sequence and the ligated primer.

Inhibition of immune function may be achieved by inducing anergy of specific T cells, or by ablation of reactive T cells. The subject binding complexes allow a therapy to be targeted to very specific subsets of T cells. The ability to inhibit immune system functions is known to be therapeutically useful in treating a variety of diseases such as atherosclerosis, allergies, autoimmune diseases, certain malignancies, arthritis, inflammatory bowel diseases, transplant rejection and reperfusion injury. Specific diseases of interest include systemic lupus erythematosus; rheumatoid arthritis; polyarteritis nodosa; polymyositis and dermatomyositis progressive systemic sclerosis (diffuse scleroderma); glomerulonephritis; myasthenia gravis; Sjogren's syndrome; Hashimoto's disease; Graves' disease; adrenalitis; hypoparathyroidism; pernicious anemia; diabetes; multiple sclerosis, and related demyelinating diseases; uveitis; pemphigus and pemphigoid cirrhosis; ulcerative colitis; myocarditis; regional enteritis; adult respiratory distress syndrome; local manifestations of drug reactions, such as dermatitis, etc.; inflammation-associated or allergic reaction patterns of the skin; atopic dermatitis and infantile eczema; contact dermatitis; psoriasis; lichen planus; allergic enteropathies; allergic rhinitis; bronchial asthma; transplant rejection, *e.g.* heart, kidney, lung, liver, pancreatic islet cell, etc.; hypersensitivity or destructive responses to infectious agents; poststreptococcal diseases, e.g. cardiac manifestations of rheumatic fever, and the like.

To ablate specific T cells, the subject binding complexes may be conjugated to a toxin moiety. Various cytotoxic agents are known and have been used in conjunction with specific binding molecules. Illustrative of these compounds are ricin, abrin, diphtheria toxin, maytansinoids, cisplatin, and the like. Where there are two subunits, only the cytotoxic subunit may be used, *e.g.* the α-unit of ricin. The toxin is conjugated to the binding complex, generally by means of a cross-linker, or other conjugation that includes a disulfide bond. Toxin conjugates are disclosed in U.S.

Patent no. 5,208,020; U.S. Patent no. 4,863,726; U.S. Patent no. 4,916,213; and U.S. Patent no. 5,165,923. The toxin conjugate is administered so as to specifically eliminate the target T cells without exerting significant toxicity against other cells.

The subject binding complexes may be administered to a host to induce anergy of the specific T cells. The binding complex will induce T cell anergy upon binding, because the co-stimulatory molecules necessary for T cell activation are not present. The binding complexes are administered to individuals, preferably mammals, in a manner that will maximize the likelihood of the binding complexes reaching the targeted T cell and binding to it, and thereby inducing anergy. This in turn will inhibit the immune response mediated by that T cell.

The dose for individuals of different species and for different diseases is determined by measuring the effect of the binding complexes on the lessening of these parameters which are indicative of the disease being treated. The binding complexes will normally be administered parenterally, preferably intravenously. Doses of binding complexes in a mouse model will generally range from about 0.5-2 mg/host/week for from about 1 to 4 weeks. The dose of the binding complexes may have to be repeated periodically depending upon the particular disease.

When administered parenterally, the binding complexes will be formulated in an injectable dosage form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and Hanks' solution. Non-aqueous vehicles such as fixed oils and ethyl oleate may also be used. The vehicle may contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability, e.g. buffers and preservatives. The binding complexes is preferably formulated in purified form substantially free of aggregates and other proteins at concentrations of about 1-50 mg/ml. Suitable pharmaceutical vehicles and their formulations are described in

Remington's Pharmaceutical Sciences, by E.W. Martin, which is incorporated herein by reference.

The following examples are offered by way of illustration and not by way of limitation.

A tetrameric MHC antigen-peptide complex can be synthesized as follows.

Production of a tetrameric MHC protein-peptide binding complex reagent is performed as follows. Briefly, plasmid vectors were constructed to express a fusion protein of a *BirA* substrate peptide (BSP) (see Shatz *et al.* [1993]) joined to one of the chains of a class II MHC molecule. The α chain of the murine I-E^k protein was used.

The fusion protein, termed *Ec*-I-E^k_α-BSP, was expressed in *E. coli*. The fusion protein was then folded *in vitro* with *Ec*-I-E^k_β and the αβ heterodimers purified on an immunoaffinity column (described in Altman *et al.* [1993] *supra*. and Wettstein *et al.* [1991] J. Exp. Med. 714:219-228). The heterodimers were biotinylated *in vitro* with purified BirA enzyme. Specific antigenic peptides are then inserted into the MHC heterodimer. The complex is bound to streptavidin previously labeled with Texas Red, and purified by gel filtration chromatography.

A plasmid for expression of Ec-I-E^k_α BSP can be constructed as follows.

Using the polymerase chain reaction, DNA coding for the 15 residue BirA substrate peptide (BSP) (SEQ ID NO:1) LHHILDAQKMVWNHR, was fused (Schatz, 1993) to the 3' end of a previously described gene for the expression of soluble I-E^k_α in *E. coli* (Altman, 1993). An antisense oligonucleotide was designed (SEQ ID NO:2) 5' CCGGAATTCTTAACGATGATTCCACACCATTTTCTGTGCATCCAGAATAT-GATGCAGGAGGAGGGTTTTCTCTTC 3'. In the sense direction the oligonucleotide provides for the 18 bases corresponding to the C-terminus of soluble *Ec*-I-E^k_α, 45 bases encoding the BSP, a stop codon and an *Eco*R I restriction site, plus

flanking bases. The sense primer for the PCR, (SEQ ID NO:3) CATATGGCTA-GCATCAAAGAGGAACACACCAT has been previously described (Altman, 1993).

The gene coding for the *Ec*-I-E^{k}_{α}-BSP fusion was amplified using the *Ultma* enzyme (Perkin-Elmer/Cetus) according to instructions from the manufacturer, with the supplied buffer components. The amplification mixes contained 50 μM of each dNTP, 1.5 mM $MgCl_2$ and 0.1 μg of the pGEM-EAK plasmid as target DNA. The reaction mixes were subjected to three cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min. The annealing temperature was then increased from 45°C to 55°C for 12 additional cycles, followed by a final incubation at 72°C for 7 minutes.

The PCR product was digested with NheI and EcoRI and was ligated into the pGEMEX-1 plasmid (Promega) digested with the same enzymes. The plasmids were transformed into the *E. coli* strain JM109 and clones containing the correct sequence were identified by sequencing of double strand plasmid DNA with Sequenase (Amersham/USB). A plasmid containing the correct sequence for the *Ec*-I-E^{k}_{α}-BSP gene was used to transform the BL21(DE3) pLysS strain of *E. coli* for expression of the fusion protein.

Ec-I-E^{k}_{α}-BSP expression and *in vitro* folding can be achieved as follows.

Inclusion bodies containing the *Ec*-I-E^{k}_{α}-BSP protein were produced following established protocols (Altman *et al.* [1993]), with the following modifications. Following expression, the protein was solubilized in a buffer lacking reducing agents (*e.g.* dithiothreitol or mercaptoethanol) but containing all previously described components (6M guanidinium hydrochloride, 50 mM Tris, pH 8.0, 1 mM EDTA). The protein concentration was kept at or below 1 mg/ml, and disulfides were allowed to form by air oxidation over 3-5 days while the protein was still in denaturant. This modified protocol was also followed for the *Ec*-I-E^{k}_{β} chain.

Soluble *Ec*-I-E^{k} heterodimers were produced in large scale *in vitro* folding reactions as described (Altman, 1993), except that glutathione and peptide ligands were

not included in the folding reactions. Empty *Ec*-I-E^k α–BSP/β heterodimers were isolated by immunoaffinity chromatography on a column containing the anti-I-E^k monoclonal antibody 14-4-4S as described (Wettstein, 1991). Column elution buffer was exchanged for PBS by diafiltration in Centricon 30 ultrafiltration devices (Amicon).

Enzymatic biotinylation of Ec-I-E^k-BSP can be achieved as follows.

The plasmid pJS169 is a pACYC184 derivative that directs overexpression of BirA under the control of the *tac* promoter. Following expression and bacterial cell lysis, the BirA protein was purified by column chromatography, first on Fast Flow Blue Sepharose, and then by ion exchange chromatography on a MonoS column (Pharmacia). Each batch of BirA is titered for activity in a reaction mix containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM ATP, 100 µM biotin, and 2.3 µM *Ec*-I-E^k-BSP. Reactions are incubated for 16 hours at 37° C. Following removal of free biotin by diafiltration on Centricon 30 concentrators, the biotinylation level is assessed by a combination of precipitation with streptavidin-agarose (Sigma) and quantitation of non-precipitible I-E^k by sandwich ELISA. Several dilutions of biotinylated *Ec*-I-E^k-BSP are incubated with a small volume of streptavidin-agarose beads overnight at 4°C in a buffer containing 2% BSA and 5 mM sodium azide in PBS. Supernatants from these mixes, as well as from control mixes containing unmodified agarose beads, are then assayed by ELISA.

For the ELISA, Immulon-4 plates are coated overnight at 4°C with 14-4-4S at a concentration of 3.3 µg/ml in PBS. The plates are blocked with 2% BSA in PBS buffer for 1 hour at room temperature. Serial dilutions of solutions containing *Ec*-I-E^k-BSP, as well as a standard containing a known concentration of *Ec*--I-E^k, are incubated on the plate for 1-2 hours at room temperature. After washing of the plate with wash buffer (0.25% BSA / 0.05% Tween20 / 5 mM sodium azide), a rabbit anti-I-E^k antisera was applied at a dilution of 1:4000 to 1:10,000 for 1 hour. Finally, the assay was

developed by addition of a mouse absorbed, goat-anti-rabbit-alkaline phosphatase conjugate (incubation time 1 hour) and addition of substrate (Sigma AP 104 tablets). Typically, 90-100% of the I-E^k is removed from the solutions by precipitation with streptavidin-agarose, reflecting nearly complete biotinylation of the *Ec*-I-E^k-BSP protein. *Ec*-I-E^k proteins lacking the BSP are not biotinylated by this protocol, strongly suggesting biotinylation at a single site in the BSP.

Loading of the bio-Ec-I-E^k-BSP with peptide can be achieved as follows.

Enzymatically biotinylated proteins are loaded with the 88-103 peptide from moth cytochrome C, SEQ ID NO:4, ANERADLIAYLKQATK according to established protocols (Wettstein [1991], *supra*; Reay [1992] *supra*). The empty αβ heterodimer was incubated with McIlvaine's citric acid-phosphate buffer (CPB) at 37°C in presilanized microfuge tubes. Reactions were adjected to pH 7 by addition of 2 M Na_2HPO_4. Peptide loaded bio-*Ec*-I-E^k-BSP molecules are purified by gel filtration on Superdex 200 columns, in a PBS mobile phase.

Production of bio-Ec-I-E^k-BSP tetramers can be achieved as follows.

Tetramers were produced by mixing the bio-*Ec*-I-E^k-BSP molecules with streptavidin-Texas Red (Boehringer Mannheim) at a 4:1 molar ratio. To maximize production of tetramers, the desired quantity of streptavidin is added in 10 equal steps, with an incubation time of 15 minutes between additions. This ensures that nearly all of the streptavidin added at each addition is saturated with bio-*Ec*-I-E^k-BSP, and after the tenth addition, the reaction mixture contains predominantly bio-*Ec*-I-E^k-BSP tetramers, and small amounts of excess streptavidin and any *Ec*-I-E^k-BSP protein that was not biotinylated by BirA.

The streptavidin-TR binding reactions are first concentrated in Centricon 30 devices, and the minor contaminants are easily removed by gel filtration on Superdex 200 columns. The tetramer elutes from the column in an early peak, corresponding to a

molecular weight of approximately 250,000, while the contaminants have molecular weights near 50,000. The labeled tetramers are concentrated to a concentration of 3-4 mg/ml and are stored in PBS + 5 mM sodium azide at 4°C in amber vials prior to use.

Labeling of antigen-specific T cells with a tetrameric MHC antigen-peptide complex can be achieved as follows.

Cells from the draining lymph nodes of a 5C.C7 T cell receptor (TCR) transgenic mouse are mixed with lymph node cells from a non-transgenic AKR/J mouse at varying ratios. The transgenic mouse is described in Seder *et al.* (1992) J. Exp. Med. 176:1091-1098. It is transgenic for the α and β chain of the 5C.C7 T cell receptor. The 5C.C7 mouse has a B10.BR genetic background, which is I-E^k and Thy 1.2 positive, while the AKR/J strain is also I-E^k positive, but is Thy 1.2 negative. The Thy 1 allotypic marker therefore allows easy identification of T cells from the transgenic mouse. FACS plots display cells which are gated to be CD4+ and negative for CD8, B220, and Mac-1 using the Desk software at the Stanford FACS facility, data shown in Figure 1.

The antibodies and concentrations used in the staining experiments are listed in Table 1.

Table 1

Antigen	Staining Reagent	Label	Supplier	Final Concentration
CD4	YT5 191.1	APC	Caltag	10 µg/ml
CD8	53-6.7	PE	Pharmingen	10 µg/ml
B220	RA3-6B2	PE	Pharmingen	10 µg/ml
Mac-1	M1/70	PE	Pharmingen	10 µg/ml
Thy 1.2	53-2.1	FITC	Pharmingen	10 µg/ml
	Streptavidin	Texas Red	Boehringer-Mannheim	10 µg/ml
TCR	Binding complex	Texas Red	-	70 µg/ml

2 x 10^6 cells, at a concentration of 4 x 10^7 cells/ml were incubated with the staining reagent for 30-60 minutes at 4°C. The cells were washed once with PBS + 2% FCS prior to analysis. The data shown in Figure 1 results from the analysis of 5 x 10^5 cells. The data in Figure 2 was obtained from analysis of 5 x 10^4 cells.

The mixing experiment demonstrates that the multimeric labeling reagent will bind to the transgenic cells, even in a large background of non-transgenic cells, many of which also recognize peptides bound to I-E^k.

The staining with the subject labeling reagent was shown to be specific for the transgenic T cell receptor by the following experiment. 5C.C7 transgenic lymph node cells were preblocked with KJ25 monoclonal antibody, which is specific for the TCR $V\beta3$ variable regions (The 5C.C7 TCR transgene is $V\beta3$ / $V\alpha11$). These cells were then stained with the subject labeling reagent. The data is shown in Figure 2. The displayed cells were gated to be positive for CD4 and negative for B220, CD8 and Mac-1. The KJ25 antibody completely blocked binding of the MHC reagent at the higher concentration.

It is evident from the above results that the subject invention provides for a novel method of specifically labeling T cells according to their antigen receptor. Stable multimeric complexes of MHC proteins and specific antigenic peptides are able to bind to T cells recognizing the complex, allowing the labeling, analysis and separation of the particular T cell subset. The invention allows for the tracking and separation of specific T cells.

Construction of a diphtheria toxin binding mutant was carried out as follows.

In order to use the Diphtheria Toxin (DT) moiety in association with soluble 'trimer' of MHC Class 1 molecules it is first necessary to mutate DT so that it is unable to bind to natural recptor (HB-EGF Precursor). The mutated protein must still be able to translocate across a biological membrane (the early endosomal membrane), and also retain its catalytic activity (ADP-ribosylation of EF-2).

The crystal structure of DT, solved to 2.3A, revealed that the toxin consists of three domains corresponding to the three basic steps in the action of the toxin: the C domain (catalytic, amino-terminal), the T domain (transmembrane, intermediate location), and the R domain (receptor binding, carboxyl-terminal). Initial identification of the regions involved in receptor interaction came from various studies using peptide fragments of DT that exhibit receptor-blocking activity and from mutations that affect receptor binding. As a whole these findings indicated that the receptor-binding site of DT lay within the toxin's carboxyl-terminal 54 residues. However, no specific residues within this region were identified. There is strong evidence that 2 specific residues, Lys-516 and Phe-530, were involved in receptor recognition, and 4 additional residues were implicated as playing lesser roles. Mutations of both of these residues to an Ala residue (K516A and F530A), resulted in significant reductions in the ability of the mutated toxin to compete with radio-labelled wild-type toxin for receptors. K516A caused a -500-fold reduction, and F530A gave a ~100-fold reduction.

It has now been shown that the glutamic acid at position 141 of the DT receptor is critical for toxin binding. Mutating Glu 141 to His 141 gave a ~100-fold reduction in binding of the receptor to the toxin. Glu 141 of the receptor interacts with Lys 516 of DT. A complex of DT with an extracellular fragment of the HB-EGF precursor receptor was crystallised and the structure solved to 2.65A resolution.

From this structure it seemed that the Glu at position 141 in the receptor could interact with the Lys at 516 of the toxin. Mutation at Lys 516 to an Ala resulted in a ~500-

fold reduction in binding.

The Phe at position 530 in DT was also implicated in having a binding role. The exact interaction of the receptor and the toxin in this region is still unclear.

In addition the serine at position 147 in the receptor is considered to interact with the Phe at 530 of DT. The complete R Domain, or just part of it, could be deleted, however mutation of the residues involved in receptor binding has been considered to be more effective, thereby maintaining a structure that is as near wild-type as possible.

The mutations that have been made are at Lys 516 and Phe 530 both to Ala to give a double mutant that has negligible binding to its native receptor.

Mutations were performed using degenerate primers so that the following replacements could be obtained.

	Position 516	Position 530
WT	**Lys**	**Phe**
F530A	**Lys**	Ala
F530S	**Lys**	Ser
(F530V)	**Lys**	Val
K516A	Ala	**Phe**
K516A; F530A	Ala	Ala
K516A; F530S	Ala	Ser
(K516A; F530V)	Ala	Val
K516E	Glu	**Phe**
K516E; F530A	Glu	Ala
K516E; F530S	Glu	Ser
(K516E; F530V)	Glu	Val
(K516T)	Thr	**Phe**
(K516T; F530A)	Thr	Ala
(K516T; F530S)	Thr	Ser
(K516T; F530V)	Thr	Val

The double mutations shown in **Bold** have been shown to be particularly effective and their respective single mutations reduced binding. The mutations shown in brackets are a result of the degenerate primers used, these also yield effective mutants.

The complete protein contained the following:

1) Amino-terminal 6x His-Tag (purification)
2) Active site mutation (E148S) to allow expression of full-length DT. This site is reverted to wild-type after receptor binding ability has been abolished.
3) Mutation at 516 and 530 (reduce/knock out binding)
4) Carboxyl-terminal BirA-Tag (attachment to the 'trimer' complex)

The construct was expressed from the vector pET-15b (which contains a N'-His-Tag) in BL21 (DE3) *E.coli* cells.

Binding assays were performed studying mutant DT in competition with radio-labelled DT. Activity assays were performed by acid-shock of target cells to allow translocation across the plasma membrane.

This description of the invention is merely illustrative thereof and it should therefore be appreciated that various variations and modifications can be made without departing from the spirit or scope of the invention as set forth in the accompanying claims.

Claims

1 A method comprising binding a moiety to a cell at a temperature of greater than 5°C, wherein the moiety binds to a receptor on said cell that recognises an MHC peptide complex.

2 A method according to claim 1, wherein said temperature is from 30 to 38 °C.

3 A method according to claim 1 or claim 2 that includes a step of detecting that binding of the moiety to the cell has occurred.

4 A method according to any preceding claim, wherein the temperature is lowered following and a step is included to remove cells with receptors binding to moieties at a lower specificity than binding of said moiety to said cell.

5 A method according to claim 4, wherein said step comprises performing one or more washes.

6 A method according to any preceding claim that includes sorting cells into different types.

7 A method according to any preceding claim, wherein the cell binding to the moiety is amplified.

8 A method according to any preceding claim, wherein the moiety comprises an active agent.

9 A method according to claim 8, wherein the active agent is a therapeutic agent or a diagnostic agent.

10 A method according to claim 8, wherein the active agent is a toxin or an immunosupressant .

11 A method according to claim 8, wherein the active agent is a label .

12 A method according to claim 8, wherein the active agent is an agent that is active when internalised by said cell.

13 A method according to claim 12 wherein the active agent is only active when internalised or has increased activity when internalised.

14 A method according to any preceding claim, wherein said cell is a T cell.

15 A method according to claim 14, wherein said cell is a cytotoxic T cell.

16 A method according to any preceding claim that is performed *in vitro*.

17 The use of a method according to any of claims 1 to 16 in achieving internalisation of an active agent.

18 The use of a method according to any of claims 1 to 16 in screening for a therapeutically active cell.

19 A cell that has been screened by a method according to claim 18 or a cell derived from such a cell by amplification.

20 The use of a method according to any of claims 1 to 16 in screening for a harmful cell.

21 The use of a method according to any of claims 1 to 16 in killing a harmful cell or in rendering a harmful cell less harmful.

22 A moiety comprising a first part that binds to a receptor that recognises an MHC peptide complex and a second part comprising an active agent that is internalised by a cell expressing said receptor after said first part binds to said receptor.

23 A moiety according to claim 22, wherein said agent only becomes active after internalisation, or has increased activity after internalisation.

24 A moiety according to claim 22 or 23 which comprises a toxin molecule.

25 A moiety according to claim 24 wherein the toxin molecule is diphtheria toxin molecule.

26 A moiety according to claim 24 or 25 wherein the toxin molecule is modified so that its ability to bind with its native receptor is negligible.

27 A moiety according to claim 25 or 26 wherein the diphtheria toxin molecule is modified by mutation of one or more amino acids at sites selected from Lys 516 or Phe 530.

28 A moiety according to claim 27 wherein Lys 516 and/or Phe 530 are modified to an amino acid selected from the group consisting of Ala, Glu, Thr, Ser and Val.

29 A moiety according to claim 27 or 28 wherein Lys and Phe 530 are modified to Ala.

30 The use of a moiety according to any one of claims 22 to 29 in achieving internalisation of an active agent.

31 A kit comprising a moiety as described in any one of claims 1 or 22 to 29,

wherein the kit comprises instructions to use said moiety at a temperature within the range given in claim 1 or claim 2.

32 An apparatus for performing a method as described in any of claims 1 to 16.

33 A kit comprising an apparatus according to claim 26 and a moiety as described in any one of claim 1 or claims 22 to 29.

34 A moiety according to any one of claims 22 to 29, or a cell according to claim 19, for use in medicine.

35 The use of a moiety according to any one of claims 22 to 29, or a cell according to claim 19, in the preparation of a medicament for treating a disorder involving T cells.

36 The use of a moiety according to any one of claims 22 to 29, or a cell according to claim 19, in the preparation of a medicament for treating a viral, protozoan, bacterial, or immune disorder, or for treating cancer.

37 The use according to claim 36, wherein the immune disorder is an autoimmune disorder or an undesired rejection of transplanted material.

38 A pharmaceutical composition comprising a moiety according to any one of claims 22 to 29, or a cell according to claim 19.

39 The invention substantially as hereinbefore described.

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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WO 01/24829 A3

(54) Title: BINDING AGENTS

(57) Abstract: MHC peptide complexes or derivatives thereof can bind to T cell receptors in a specific manner at much higher temperatures than previously disclosed. Active agents linked to such complexes, or to derivatives thereof, can be internalised by T cells.

Figure 1

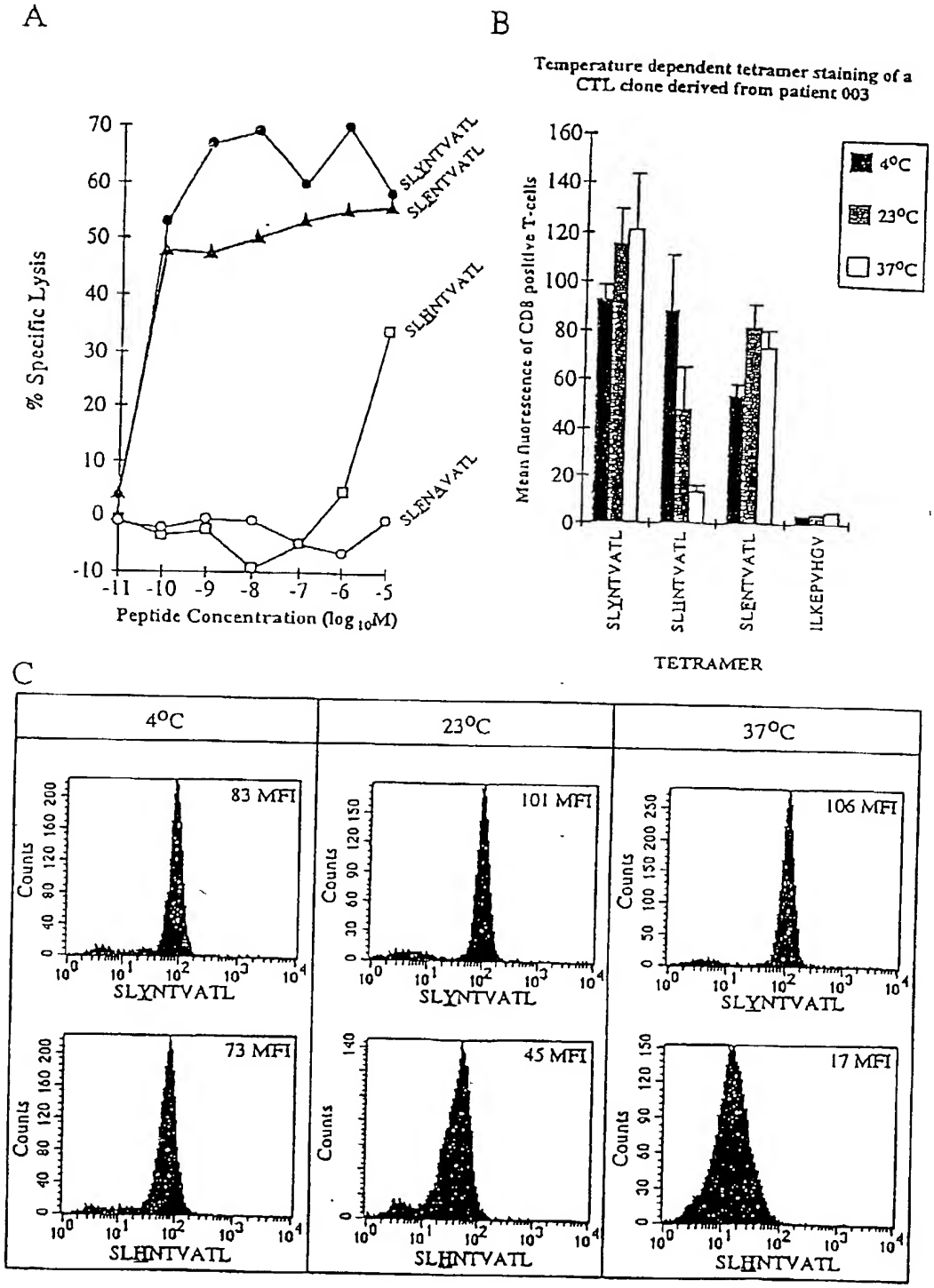

A
% Specific Lysis
Peptide Concentration (log10M)
SLYNTVATL
SLENTVATL
SLHNTVATL
SLENAVATL
B
Temperature dependent tetramer staining of a CTL clone derived from patient 003
Mean fluorescence of CD8 positive T-cells
4°C
23°C
37°C
SLYNTVATL
SLHNTVATL
SLENTVATL
ILKEPVHGV
TETRAMER
C
4°C
23°C
37°C
83 MFI
101 MFI
106 MFI
73 MFI
45 MFI
17 MFI
Counts
SLYNTVATL
SLHNTVATL

Figure 2

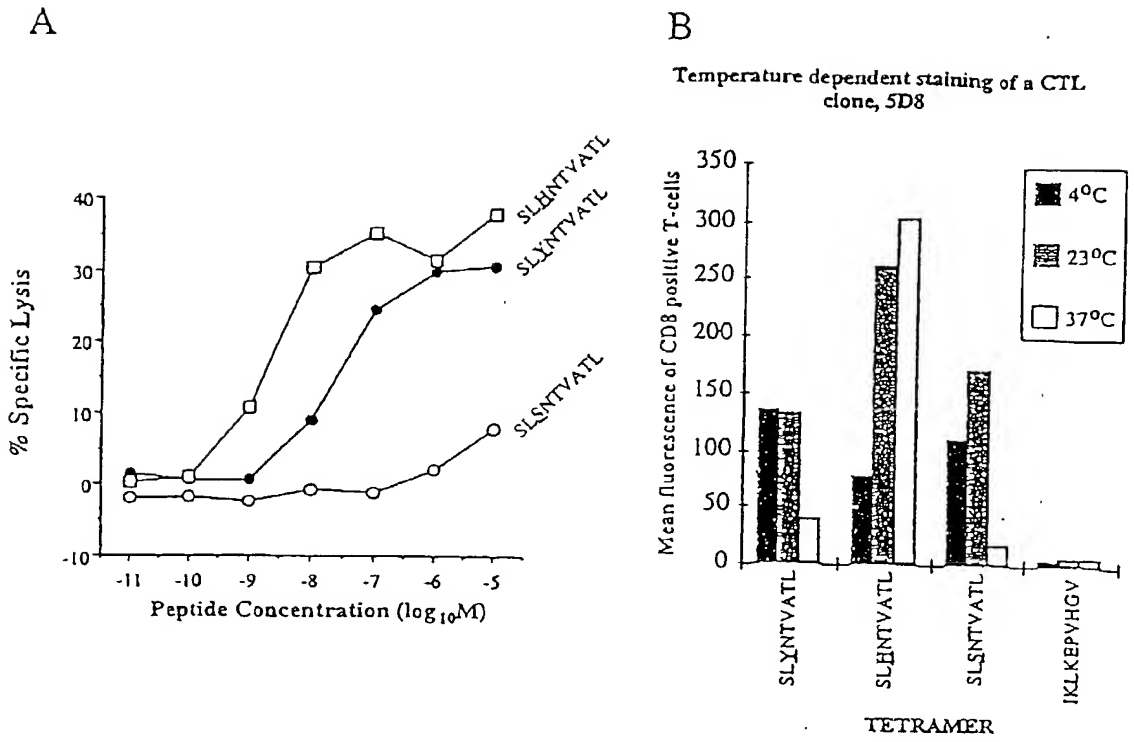

A
% Specific Lysis
40
30
20
10
0
-10
-11
-10
-9
-8
-7
-6
-5
Peptide Concentration (log_{10}M)
SLHNTVATL
SLYNTVATL
SLSNTVATL
B
Temperature dependent staining of a CTL clone, 5D8
Mean fluorescence of CD8 positive T-cells
350
300
250
200
150
100
50
0
4°C
23°C
37°C
SLYNTVATL
SLHNTVATL
SLSNTVATL
IKLKEPVHGV
TETRAMER

Figure 3

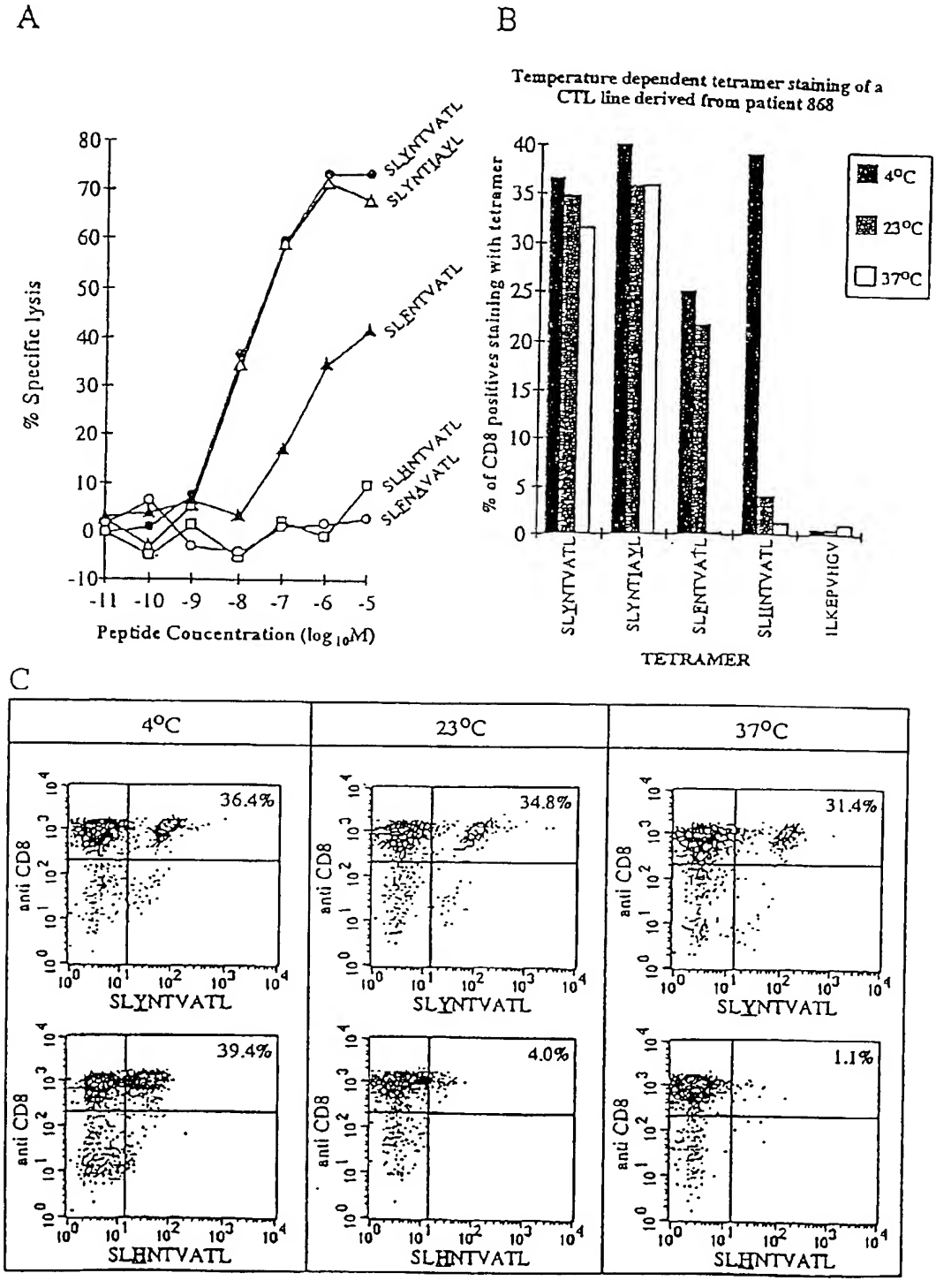

A
% Specific lysis
Peptide Concentration (log_{10}M)
SLYNTVATL
SLYNTIAYL
SLENTVATL
SLHNTVATL
SLENAVATL
B
Temperature dependent tetramer staining of a CTL line derived from patient 868
% of CD8 positives staining with tetramer
4°C
23°C
37°C
SLYNTVATL
SLYNTIAYL
SLENTVATL
SLHNTVATL
ILKEPVHGV
TETRAMER
C
4°C
23°C
37°C
anti CD8
SLYNTVATL
SLHNTVATL
36.4%
34.8%
31.4%
39.4%
4.0%
1.1%

Figure 4

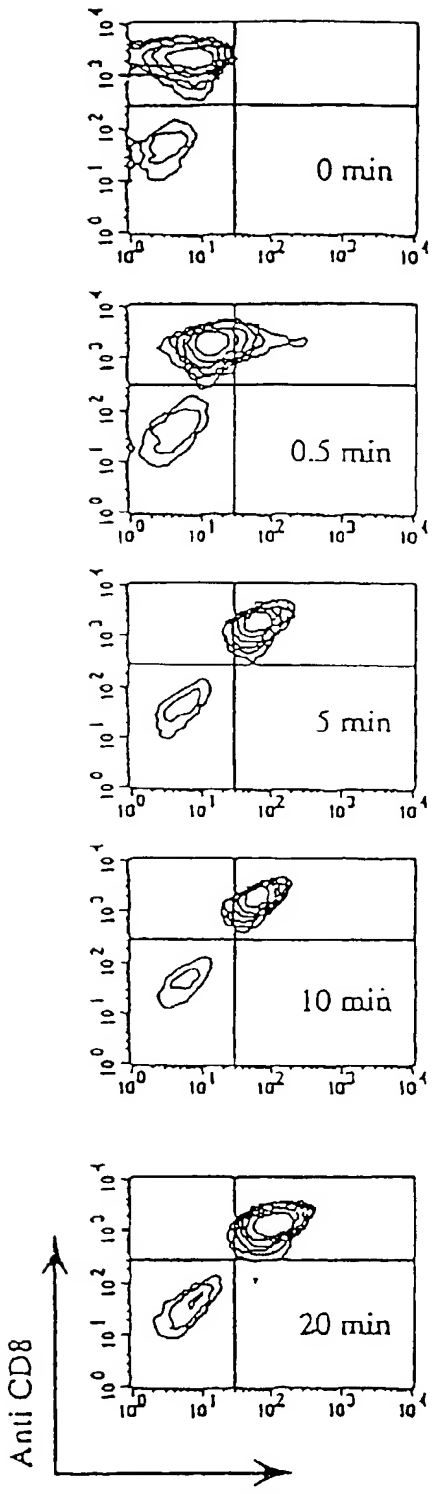
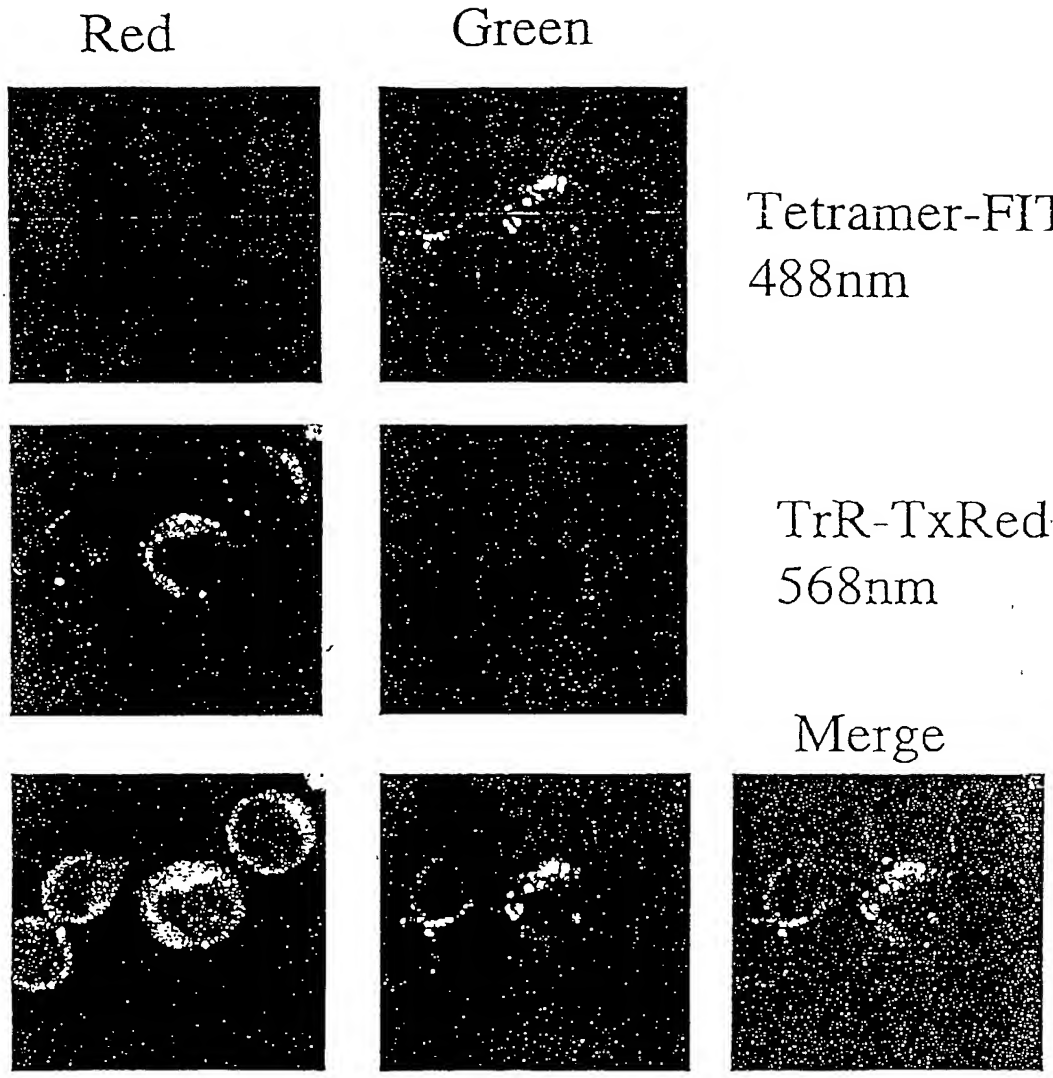


Figure 5



DECLARATION, PETITION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Check one):

- ☐ Declaration Submitted with Initial Filing
- ☒ Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BINDING AGENTS

the specification of which (check one):

☐ is attached hereto.

OR

☒ was filed on **04 October 2000** as PCT International Application Number **PCT/GB00/03792** and filed as

- ☐ and was amended by PCT Article 19 Amendment on _____ (if applicable),
- ☐ and was amended by PCT Article 34 Amendment on _____ (if applicable).

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

Atty Docket No.: **FHW-102US**

DECLARATION, PETITION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(Check one):

☐ Declaration Submitted with Initial Filing

☒ Declaration Submitted after Initial Filing

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

BINDING AGENTS

the specification of which (check one):

☐ is attached hereto.

OR

☒ was filed on **04 October 2000** as PCT International Application Number **PCT/GB00/03792** and filed as **USSN 10/089922**

☐ and was amended by PCT Article 19 Amendment on _____ (if applicable),

☐ and was amended by PCT Article 34 Amendment on _____ (if applicable).

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

PRIORITY CLAIM

(Check one):

☐ no such applications have been filed.

☒ such applications have been filed as follows

1) FOREIGN PRIORITY CLAIM: I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (dd,mm,yyyy)	Priority Not Claimed	Certified Copy Attached Yes	Certified Copy Attached No
9923337.1	**GB**	**04 October 1999 (04.10.1999)**	☐	☐	☒
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority sheet attached hereto.

2) PROVISIONAL PRIORITY CLAIM: I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Provisional Application Number(s)	Filing Date (dd/mm/yyyy)

☐ Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

3) U.S./PCT PRIORITY CLAIM: I hereby claim the benefit under Title 35, United States Code, §120 of any United States application or §365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (dd/mm/yyyy)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

POWER OF ATTORNEY:

As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Direct Telephone Calls to: (name and telephone number)

Anthony A. Laurentano, (617) 227-7400

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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400

Full name of fourth inventor **Andrew SEWELL**
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